IDENTIFICATION, CHARACTERIZATION, AND POSSIBLE FUNCTIONS DURING EARLY PREGNANCY OF UTERINE-DERIVED PEPTIDE GROWTH FACTORS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1992

To my mother and late father whom I respect the most

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Frank A. Simmen, for introducing me to the PIG world, and for support, guidance, and encouragement during my Ph.D program. Also, none of these studies would have been possible without much guidance and support from Dr. Rosalia C. M. Simmen. I would also like to thank the other members of my committee, Drs. Fuller W. Bazer, William C. Buhi, and William W. Thatcher for their scientific advice and discussion throughout my studies.

I would like to acknowledge Dr. Peter J. Hansen for access to his lab facilities. My special thanks go to all the graduate students and biologists in the laboratories of Drs. Fuller W. Bazer, Rosalia C. M. Simmen, and William W. Thatcher for their help in surgery, for sharing scientific ideas and wonderful times, and for unforgettable English lessons. I am very grateful to late Cheryl Feinstein for her assistance with lab chores. I thank Drs. Michael T. Moser and Young Lee for useful feeedback and for sharing all the hard times. I also thank Dr. Mark Mirando and Matthew Davis for their help in oTP-1 assays, Jessee Johnson for his assistance with HPLC system, and Leslie Smith with immunohistochemical studies. I appreciate all the past and present graduate students in the Dairy Science department for making my study more enjoyable.

I would like to express my deepest appreciation to all of my family members: especially, my late father who cannot see this dissertation, and my mother, sister and brother for their support, encouragement and discipline which make me what I am today. My warm thanks go to my daughter, Alice, for giving me a joyful life and smile. Lastly, I do not know how to thank my lovely wife, Eonjoo, for everything she has done for me up to now.

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May 1992

Chairperson: Dr. Frank A. Simmen
Major Department: Animal Science

To elucidate the biochemical nature of uterine-derived peptide growth factors and their potential involvement in regulation of endometrial and conceptus development, studies on the identification, characterization, and biological roles during early pregnancy of uterine-derived peptide growth factors were conducted.

A mitogenic factor, termed uterine luminal fluid mitogen (ULFM), was identified in porcine ULFs and was partially purified and characterized. ULFM is a heat-stable, small M_r (4,800) polypeptide with a pI of 6.4 and is not a pregnancy-specific protein. ULFM is apparently distinct from other known peptide growth factors based on a number of immunological and biochemical criteria. Further, ULFM is a potent mitogen for cells of embryonic and uterine origins, indicating its probable regulatory roles in the female reproductive tract during early pregnancy. Sheep ULFs were also

analyzed with respect to peptide growth factor content. ULFs from cyclic and pregnant ewes contain IGF-I and IGF-II, the levels of which vary depending on the day of estrous cycle or pregnancy. Further, ovine ULFs contain two additional, as yet unidentified, mitogenic factors, but do not contain PDGF or porcine ULFM-like activities. The in vitro secretion of ovine trophoblast protein-1 was stimulated or at least maintained by the combination of IGF-I and IGF-II, indicating a regulatory role for IGFs during the period of maternal recognition of pregnancy in sheep. In vitro effects of peptide growth factors on growth and differentiation of rabbit endometrial epithelial cells (HRE-H9) transformed with an origin-defective, temperature-sensitive SV40 mutant at 33°C were also examined. IGF-I, IGF-II, EGF, and the FGFs stimulated DNA synthesis of normal HRE-H9 cells; however, these effects were antagonized by TGF-β1. The synergistic effects of IGF-I and EGF on DNA and protein synthesis of HRE-H9 cells were observed. The mitogenic actions of IGFs on HRE-H9 cells were mediated by Type I IGF receptors. The secretion of a putative IGFBP-1 with a M_r of 31,000 was enhanced in normal HRE-H9 cells relative to transformed HRE-H9 cells.

The temporally regulated expression of peptide growth factors in ULF during early pregnancy and their complex regulation of cellular growth and differentiation highlight the potential importance of peptide growth factors as molecular mediators of coordinate conceptus and uterine development during the critical early period of pregnancy.

CHAPTER 1
LITERATURE REVIEW

Introduction

The establishment and maintenance of pregnancy requires complex and precise interactions among biochemical, immunological, and hormonal factors. Prenatal mortality during early pregnancy is a significant problem in all mammalian species. In particular, porcine embryos exhibit a high degree of mortality during early pregnancy. The molecular mechanisms underlying this reproductive problem remain to be elucidated.

Pigs, sheep, cows, and horses exhibit nonivasive types of placentation in which embryos are dependent upon uterine secretions as a nutritional source during early pregnancy. Uterine secretions which accumulate in the uterine lumen include a complex array of molecules ranging from inorganic ions to macromolecules, such as proteins. Several classes of proteins of conceptus and uterine origins in uterine secretions of domestic animals have been identified and characterized. These are thought to mediate not only maternal-fetal communication but also conceptus and uterine development. One class of endometrial regulatory proteins are the polypeptide growth factors. An increasing body of evidence indicating the involvement of polypeptide growth factors in reproductive function has facilitated our

understanding of the mechanism for conceptus and uterine development. However, more studies are required to clarify the nature of peptide growth factors and their involvement in endometrial and conceptus development of domestic animal species during early pregnancy. This is required to expand our current knowledge regarding the potential involvement of peptide growth factors in abnormal endometrial function and conceptus development contributing to prenatal mortality in mammals during early pregnancy. In this regard, studies on the identification, characterization, and possible functions of peptide growth factors for conceptus and uterine development during early pregnancy were conducted and are described in this dissertation. As a basis for the described experiments, the current state of research and understanding of prenatal mortality in the pig, maternal recognition of pregnancy in domestic animals, uterine secretory proteins, and uterine-derived peptide growth factors are reviewed in this chapter.

Embryonic Mortality

Litter size is an important biological variable affecting profitability in the swine industry. Genetic and environmental factors affecting litter size include ovulation rate, fertilization rate, uterine capacity, and embryonic survival. All mammalian species experience varying degrees of prenatal losses during pregnancy. In the human, approximately 20% of pregnancies end before they are clinically detected by physicians or other conventional pregnancy tests and 31% prior to term (Wilcox et al., 1988). In sheep and cattle, approximately 20-40% of ovulated eggs did not result

in successful pregnancy, and fertilization failure and embryonic losses before implantation accounted for these losses (Wilmut et al., 1986). In general, pigs exhibit fertilization rates of 95-100% with the presence of 14-16 viable embryos, but only 9-10 of these embryos survive to term and 7-8 of these piglets are weaned. Within the first 30 days of gestation, 35-45% of swine embryos are lost between Days 8-12 of pregnancy and an additional reduction of 10-20% occurs by term of pregnancy (Perry and Rowlands, 1962). The causes of prenatal mortality during early pregnancy in the pig and other species are still largely speculative.

The development of porcine embryos has been well studied (Heuser and Streeter, 1929; Perry and Rowlands, 1962; Hunter, 1974; Anderson, 1978; Bazer and First, 1983) and summarized in Table 1-1. Two to 3 days after ovulation, fertilized embryos primarily in the 4-cell stage enter the uterus, concomitant with activation of the embryonic genome (Freitag et al., 1988). In general, the morula stage (8-16 cells) is reached around Day 4, blastocyst stage (16-32 cells) around Days 5-6, and embryo hatching from the zona pellucida occurs at Days 6-7 (Stroband and Van der Lende, 1990). Shortly after hatching, pig blastocysts freely migrate in the uterine lumen as early as Day 9 and spacing ends when conceptuses (embryo and its associated membranes) elongate around Days 11-12 (Bazer and First, 1983). Around Days 11-12 and prior to attachment to uterine endometrium, pig conceptuses undergo rapid morphological change from spherical (3-10 mm diameter) to tubular (10-50 mm long) to filamentous (longer than 100 mm) shapes (Heuser and Streeter, 1929). During Days 10-12, blastocysts grow up to 30-45 mm/h by cellular remodelling and,

Table 1-1. Embryonic development in the pig.

Time (Day)	Developmental Stage
0	Ovulation (14-16 embryos)
2-3	Activation of embryonic genome (4-cells)
4	Morular (8-16 cells)
5-6	Blastocyst (16-32 cells)
6-7	Hatching
7-10	Migration
10-12	Morphological elongation
	(spherical--> ovoidal--> tubular--> filamentous form)
13-18	Attachment (noninvasive, epitheliochorial type)

during later stages (Days 14-16), by cellular hyperplasia (Geisert et al., 1982a). The biochemical factors responsible for initiation of blastocyst elongation and for conceptus hyperplastic growth remain unknown. Cellular remodelling during the initial elongation phase results from a flattening of trophectodermal cells and an increase in microvilli on the cell surfaces (Geisert et al., 1982a). Involvement of an altered F-actin cytoskeletal structure during elongation was also demonstrated (Mattson et al., 1990). Attachment by interlocking microvilli of conceptuses to uterine epithelium starts around Days 13-14 and is completed around Day 18 in the pig, which results in the formation of a noninvasive, epitheliochorial type of placentation (Perry, 1981). Thus, peri-implantation pig conceptuses are dependent upon uterine luminal fluids for nutrients and other essential factors for development.

A wide range of morphological stages of development exists among littermate pig embryos during early pregnancy (Anderson, 1978). For example, on Day 11 of gestation, embryos within a litter may exhibit spherical, ovoidal, tubular, and filamentous forms. Although the exact mechanism is not known, potential contributors to morphological variation among littermate embryos include asynchronous follicular oocyte maturation, differential release of oocytes during the long period (6 h) of ovulation, genetic factors, differential timing of fertilization, differential timing of gene activation and cleavage/divisions, differential oviductal transport of embryos, and different uterine locations (Pope et al., 1990). This variation in morphological development has been postulated to contribute to subsequent embryonic mortality (Polge, 1982). Therefore, if developmental variation among littermate embryos was reduced, then selection against less developed embryos might be reduced and litter size increased. Because these relationships affect embryonic survival, it is important to understand the mechanisms responsible for variation in embryonic development.

Pig blastocysts synthesize and secrete estradiol on Days 11-12 (Perry et al., 1973; Gadsby et al., 1980) which induces release of calcium (Geisert et al., 1982b; Young et al., 1987), proteins (Godkin et al., 1982a; Fazleabas et al., 1983), prostaglandins (Zavy et al., 1980), and peptide growth factors (Simmen and Simmen, 1990) from uterine endometrium. Exposure to estradiol was also shown to alter the pattern of uterine secretory proteins (Geisert et al., 1982c; Morgan et al., 1987), to induce sequestration of intrauterine $PGF_2\alpha$ (Frank et al., 1978), and to increase local

vascular permeability (Flint, 1981) and uterine blood flow (Ford and Magness, 1980). The uterine environment during the estrous cycle and early pregnancy is steroid-dependent (Murray et al., 1972) and is altered by the presence of conceptus-derived estrogens (Geisert et al., 1982b). In the sheep, the presence of blastocysts causes quantitative and qualitative changes in the pattern of uterine secretory proteins during early pregnancy (Findlay et al., 1982; Vallet et al., 1987; Ashworth and Bazer, 1989). Although the existence of an embryocidal effect of advanced uterine environment on less developed embryos is only presumed, it is possible that, by synthesizing estrogens earlier, the more advanced embryos create a uterine environment which is detrimental for less developed embryos. In this regard, the morphologically advanced embryos have been shown to synthesize estrogens and other biomolecules earlier than the less developed embryos (Ford et al., 1982; Geisert et al., 1982b; Pusateri et al., 1990). Furthermore, embryo transfer experiments have demonstrated that the more developed embryos have a greater chance of survival than do their less developed counterparts (Pope et al., 1982). Wilde et al. (1988) observed that the less developed embryos are more sensitive to the advancement of uterine environment than are more developed embryos. It is possible that administration of estrogens causes the premature secretion or termination of compounds essential for normal embryo development. For example, treatment of sows with estrogen at Day 9-10 of pregnancy causes changes in the profile of secreted proteins (Gries et al., 1989), which results in embryonic death. Similarly, in rodents, the pattern of endometrial secreted proteins is controlled, in

part, by ovarian steroids (Aitken, 1977; Surani, 1977) and/or embryonic developmental stages (Nieder et al., 1987). Contrary to other workers, Lambert et al. (1991) reported that most of the embryonic losses in pregnant gilts occurred before Day 10. It was postulated that the limited capacity of the porcine uterus and overcrowding by conceptuses result in a competition for critical biochemical substance(s) produced by the uterus, resulting in embryonic losses (Bazer et al., 1969). On the other hand, Polge (1982) suggested a high degree of blastocyst asynchrony rather than uterine overcrowding as the factor causing prenatal mortality. Interestingly, Trout et al. (1991) postulated that the degree of asynchrony among pig littermates might be decreased by vitamin A supplementation to less developed embryos, based on the importance of retinol availability for developing embryos in uterine secretions. Blair et al. (1991) observed that early exogenous estradiol on Days 9-10 of pregnancy resulted in total embryonic loss by Day 18, which might be caused by alterations in uterine endometrial epithelium, such as a thinning of the uterine epithelial glycocalyx and a reduction of ferritin binding to microvilli, during pig conceptus attachment. The demonstrated importance of glycans for female reproductive function (Aplin, 1991) supports this finding. These results emphasize the importance of synchronized interactions between the conceptus and uterine endometrium.

Results from embryo transfer experiments also suggest that a close developmental synchrony between the embryo and the recipient uterus must be maintained for successful implantation (Pope and First, 1985; Morgan et al., 1987; Stroband and Van

der Lende, 1990). Coculture of embryos with endometrial monolayers was beneficial to pig embryo development (Allen and Wright, 1984). In the rabbit (Chang, 1950), the timing of embryo entry into the uterus has been suggested to be critical for subsequent development.

Genetic factors can also influence embryonic development. The mouse major histocompatibility complex (MHC) (or H-2 complex) associated with preimplantation embryonic development (PED) gene (Goldbard and Warner, 1982; Warner et al., 1987) is implicated as a regulator of the cleavage rate of mouse embryos during the preimplantation period. PED genes for fast and slow cleavage rates are expressed at the time of the first cleavage division. The detection of MHC antigens (SLA; swine leucocyte antigen complex) on early pig embryos (Days 2-6) (Warner et al., 1986) and the observation of differential cleavage rates depending on SLA genotypes of preimplantation pig embryos (Days 9-11) (Ford et al., 1988) suggest the involvement of MHC genes in embryonic development and subsequent survival.

Prolific Chinese Meishan (MS) gilts are characterized by rapid growth and high prolificacy (30-40% greater than average litter size) (Bazer et al., 1988a, 1988b). Although MS pigs show a lower ovulation rate and smaller uteri compared to European Large White pigs (Bazer et al., 1988a), MS gilts exhibit higher uterine secretory activity and greater accumulation of histotroph components, such as prostaglandins, glucose, estradiol, and insulin-like growth factor-I (Simmen et al., 1989a; Bazer et al., 1991b), which support development of conceptuses between Days 8-14. Bazer et al. (1991b) suggested that the faster and more uniform conceptus

development in MS gilts resulting in increased embryo survival is possibly due to the greater accumulation of histotroph in the uterine lumen.

Based on the above observations, the developmental synchrony between the embryo and uterus appears to be a determing factor for subsequent embryonic development. A high rate of embryonic loss occurs mainly during early pregnancy when dramatic changes in the uterine environment occur. Since alterations in uterine environment is directly related to embryonic development, it is critical to analyze the nature of the biochemical factors causing these observed changes during early pregnancy.

Maternal Recognition of Pregnancy

Since the embryo is a semi-allograft, it must let the mother know of its presence so as not to be rejected as a foreign body. This mechanism should be operative at the critical time of implantation. This communication between mother and embryo is referred to as maternal recognition of pregnancy and leads to maintenance of a functional corpus luteum (CL), regulation of the maternal immune system, and regulation of nutritional availability via control of blood flow and uterine secretions. Luteostatic (as in pig) or antiluteolytic (as in cow and sheep) signals from the conceptus serve to prevent the action of luteolysin prostaglandin $F_2\alpha$ ($PGF_2\alpha$) and to maintain a functional CL for extended periods in order to provide sufficient circulating concentrations of progesterone for endometrial secretory activity. The

substances secreted by the uterus are essential for support of conceptus development and successful pregnancy.

Pigs

Estrogens secreted by pig conceptuses around Days 11-12 are believed to be responsible for maternal recognition of pregnancy (Bazer and Thatcher, 1977; Ford et al., 1982). Extended CL function by exogenous estrogens has been demonstrated (Ford and Magness, 1980; Geisert et al., 1987). Estrogens do not inhibit the synthesis of endometrial $PGF_2\alpha$ but alter the direction of $PGF_2\alpha$ secretion to protect CL integrity. Released estrogens act on uterine endometrium to cause Ca^{2+} mobilization by endometrial epithelial cells and activation of phospholipase A_2 to cause fusion of secretory vesicles with endometrial epithelial cell membranes (Rubin and Laychock, 1978). The molecules including $PGF_2\alpha$ stored within the secretory vesicles are transported into the uterine lumen via exocytosis (Geisert et al., 1982c). This is believed to cause a switch in $PGF_2\alpha$ secretion from an endocrine direction (toward the uterine vasculature) to an exocrine direction (into the uterine lumen). Sequestration of $PGF_2\alpha$ in the uterine lumen thus prevents $PGF_2\alpha$-induced CL regression (i.e., luteolysis). Higher amounts of $PGF_2\alpha$ were found in uterine flushings from pregnant gilts than in those from cyclic gilts (Zavy et al., 1980).

Pig conceptuses secrete a complex array of proteins. Two major classes of porcine conceptus-secretory proteins (pCSP) were isolated and characterized (Godkin et al., 1982a). These proteins with molecular weight (M_r) of 20,000-25,000

[isoelectric point (pI) of 5.6-6.2] and of 35,000-50,000 (pI of 8.2-9.0) are present during Days 10.5-18 of pregnancy. Although pCSP does not exhibit antiluteolytic activity (Harney and Bazer, 1989), antiviral activity due to secretion of α- and τ-interferons (LaBonnardiere et al., 1991) was found in uterine flushings and in conceptus-conditioned culture medium with peak levels at Day 16 (Mirando et al., 1990a). Porcine CSP stimulates endometrial production of $PGF_2\alpha$ and PGE_2 (Harney and Bazer, 1989). PGE_2 in turn causes vasodilation, increased uterine blood flow, and increased capillary permeability, all of which result in enhanced nutrient transport. Thus, pCSP may, in part, regulate endometrial secretory activity and conceptus growth (Harney and Bazer, 1989). These proteins might also mediate uterine expansion to accommodate developing conceptuses (Harney and Bazer, 1990).

Sheep

Since antiluteolytic effects were elicited by ovine conceptus secretory proteins (oCSP) containing oTP-1 or by oTP-1 alone (Godkin et al., 1984b; Vallet et al., 1988b), oTP-1 is considered to be the maternal recognition of pregnancy signal in the sheep. Ovine TP-1 is an acidic (pI 5.3-5.7) nonglycosylated protein (172 AA, M_r of 19,000) with at least 3 isomeric forms, is secreted in temporal fashion (Days 10-21) by conceptus trophectoderm (Godkin et al., 1982b), and has 70% amino acid sequence homology with interferon$_\alpha$-II (Imakawa et al., 1987). Complementary DNA clones for oTP-1 have been isolated (Charlier et al., 1989; Imakawa et al., 1989) and

recombinant oTP-1 produced by using a yeast expression system (Ott et al., 1991). The expression of oTP-1 mRNA was not detected until Day 12 and increased in abundance to maximal levels at Days 14-16, with concomitant maximal production of protein (Farin et al., 1989). Secreted oTP-1 modulates prostaglandin synthesis (Salamonsen et al., 1989) and endometrial protein synthesis and secretion (Godkin et al., 1984a), and shows antiluteolytic activity by extending CL life span when infused into the uterus of cyclic ewes (Godkin et al., 1984b). Moreover, oTP-1 exhibits antiviral activity (Pontzer et al., 1988) and is immunosuppressive (Newton et al., 1989) as well as antiproliferative (Pontzer et al., 1988; Roberts et al., 1989).

The mechanism of antiluteolytic action of oTP-1 has not been defined clearly. However, the secretion of oTP-1 apparently prevents the synthesis of oxytocin receptor. Follicular estrogens induce the synthesis of oxytocin receptor (McCracken et al., 1984) and oxytocin stimulates pulsatile uterine $PGF_2\alpha$ secretion (Flint and Sheldrick, 1986). The binding of oTP-1 to high-affinity, low capacity binding sites (Godkin et al., 1984a; Hansen et al., 1989; Knickerbocker and Niswender, 1989) on endometrium results in the induction of several endometrial proteins (Vallet et al., 1987; Ashworth and Bazer, 1989). Although oxytocin is still released by the CL, this does not result in release of $PGF_2\alpha$ from endometrium since oTP-1 is thought to inhibit effects of estrogen and/or progesterone on synthesis of endometrial receptors for oxytocin and/or inhibit endometrial synthesis and/or recycling of oxytocin receptors directly (Vallet et al., 1988b; Bazer, 1989). As a result, $PGF_2\alpha$-induced luteolysis is prevented. Alternatively, oTP-1 may prevent $PGF_2\alpha$ secretion by

inhibiting oxytocin-stimulated inositol phosphate turnover in endometrium during the maternal recognition of pregnancy period (Mirando et al., 1990b). It is also speculated that oTP-1 acts on endometrial epithelium to stabilize progesterone receptor and/or its mRNA and inhibit upregulation of estrogen and oxytocin receptors and/or their mRNAs (Ott et al., 1992).

Goats

Caprine trophoblast protein-1 (cTP-1) is apparently the conceptus signal for maternal recognition of pregnancy in the goat (Gnatek et al., 1989). Conceptuses between Days 16-21 secrete cTP-1 which is a complex of six nonglycosylated and glycosylated polypeptides (Baumbach et al., 1990). The most prominent protein has a M_r of 17,000 with a pI of 5.2-5.7. The observations that cTP-1 is immunoprecipitated by antiserum raised to oTP-1 (Gnatek et al., 1989) and that oTP-1 cDNA hybridizes to total RNA from Day 17 goat conceptuses (Charlier et al., 1989) suggest significant homologies between the two molecules and hence similar antiluteolytic properties.

Cows

The bovine conceptus (Days 14-25) secretes a complex of proteins, termed bovine trophoblast protein-1 (bTP-1) (Bartol et al., 1985; Helmer et al., 1987), which are the signal for maternal recognition of pregnancy in the cow. This protein is comprised of at least seven isoforms of N-linked glycoproteins differing in M_r, extent of

glycosylation (22,000 high mannose and 24,000 complex form), and isoelectric points (Anthony et al., 1988). The amino acid sequence of bTP-1 exhibits 82% homology with oTP-1 and 50% homology with IFN_α (Imakawa et al., 1989). Further, bTP-1 possesses similar biological properties to oTP-1, such as antiviral (Godkin et al., 1988), immunosuppressive and antiluteolytic (Helmer et al., 1989b) activities, and antiserum raised to oTP-1 cross-reacts with bTP-1 (Helmer et al., 1987). Using an oTP-1 cDNA probe, the presence of bTP-1 mRNA was detected in trophectoderm as early as Day 12 of pregnancy (Farin et al., 1990). The maximal expression of the bTP-1 transcript at Days 15-16 (Farin et al., 1990) and maximal bTP-1 secretion at Days 16-19 (Bartol et al., 1985) of gestation supports the importance of bTP-1 during the period of maternal recognition of pregnancy in the cow. Thatcher et al. (1989) and Helmer et al. (1989b) demonstrated that intrauterine administration of bTP-1 into cyclic cows resulted in an increase in interestrous interval, thus confirming bTP-1 as the antiluteolytic factor.

Currently, the elucidation of the mechanism of bTP-1 action to prevent luteolysis in the cow is ongoing. Although bTP-1 exhibits similar characteristics to oTP-1, its proposed mechanism of action is different. Binding of bTP-1 to receptors on the endometrium induces the synthesis of an endoplasmic prostaglandin synthetase inhibitor (EPSI: M_r of 25,000-35,000 and 70,000-75,000) (Gross et al., 1988). EPSI is proposed to block the conversion of arachidonic acid (AA) to $PGF_2\alpha$ by inhibiting cyclooxygenase activity of epithelial cells (Helmer et al., 1989a). This results in reduced secretion of luteolytic $PGF_2\alpha$ from endometrial epithelial cells (Danet-

Desnoyers et al., 1991). A report indicating that signalling by IFN_α occurs through AA hydrolysis (Hannigan and Williams, 1991) supports the possible pathway of bTP-1 action via EPSI induction. An EPSI-like inhibitory factor for prostaglandin synthesis was also identified in the endometrium of the sheep (Basu, 1989). Similarly to oTP-1 action, bTP-1 may inhibit the synthesis of oxytocin receptor since Fuchs et al. (1990) observed the low concentration of oxytocin receptor in the endometrium of early pregnant cows.

On the other hand, based on similar biological properties between bTP-1 and IFNα, a 2′,5′-oligoadenylate(A) synthetase-mediated bTP-1 action for maternal recognition of pregnancy has been proposed (Short et al., 1991). IFNs induce the activity of the enzyme 2′,5′-oligo(A) synthetase which catalyzes the production of 2′,5′-oligo(A) that is a potent antiviral and growth inhibitory molecule. Highly enriched preparation of bTP-1 and IFNs exhibited similar activities in inducing 2′,5′-oligo(A) synthetase activity from ipsilateral endometrial stromal cells from the pregnant cow, but low stimulatory activities were observed during the estrous cycle.

Mares

Maternal recognition of pregnancy in the mare occurs between Days 14-16 of gestation (Hershman and Douglas, 1979). The equine embryo migrates from one uterine horn to the other (12-14 times a day) and secretes estrogens. A restriction of that mobility led to termination of pregnancy (McDowell et al., 1988). Sharp et al. (1984) observed that intrauterine levels of $PGF_2\alpha$ were lower in pregnant than in

nonpregnant mares, but in vitro production of endometrial $PGF_2\alpha$ continued to increase after the equivalent day of luteolysis (Days 14-16) and pseudopregnancy was not induced by exogenous estrogen. Later, Sharp et al. (1989) co-cultured conceptus membranes with endometrial explants and found reduced secretion of endometrial $PGF_2\alpha$, demonstrating the involvement of an embryonic factor(s) in maternal recognition of pregnancy. However, Goff et al. (1991) observed the enhanced oxytocin stimulation of prostaglandin release by estrogens at the time of luteolysis. This indicates that estrogens are not the signal for maternal recognition of pregnancy in mares and suggests a possibility that estrogens might be a cofactor to maintain pregnancy in the mare. Recently, Watson (1991) examined the effect of addition of endometrial cytosol from pregnant and cyclic (Day 14) mares on prostaglandin synthesis by microsomes. The results suggest that the reduced secretion of $PGF_2\alpha$ during early pregnancy in the mare is due to the presence of endogenous prostaglandin synthesis inhibitors within the endometrium, which is similar to EPSI present in cow endometrium (Gross et al., 1988).

Other species

Several other embryo-derived substances from primates and rodents have been described. These include an early pregnancy factor (Morton et al., 1977), platelet-activating factor (O'Neill et al., 1989; Harper, 1989), Schwangershafts protein (Sinosich et al., 1985), and chorionic gonadotropin (Fishel et al., 1984). However, their exact roles and mechanisms of action during the early pregnancy are still poorly

understood. These molecules are speculated to facilitate processes occurring during early pregnancy, which include implantation, embryonic and uterine development, and synthesis of proteins and/or hormones, although their possible roles as signals for the maternal recognition of pregnancy are not excluded (Hearn et al., 1991; van der Weiden et al., 1991).

These embryonic signalling molecules for establishment and maintenance of pregnancy stimulate the secretion of endometrial secretory biomolecules into the uterine lumen where they come into contact with and may support the development of conceptuses. It is, therefore, important to analyze the composition of these secretory molecules and to characterize their functions, which eventually may lead to a better understanding of embryo development in utero.

Uterine Secretory Proteins

Uterine secretions, or histotroph (Grosser, 1924), are secreted from endometrial surface and glandular epithelium into the uterine lumen and constitute part of the uterine environment for developing conceptuses, similar to an in vitro culture medium for growing cells. Uterine secretory activity is mainly under progesterone control, but estrogens, via stimulation of progesterone receptor synthesis and other effects, are synergistic or additive with progesterone. The ratio of progesterone to estrogen is particularly important for determining the state of uterine growth and secretory activity. After implantation, conceptuses of humans, rodents, and rabbits exhibit hemochorial placentation by invading the uterine wall and using maternal

blood supply for nutritional support. Thus, conceptuses of these species are less dependent on uterine secretions. However, conceptuses of pigs, cows, sheep, and horses must depend on uterine secretions for nutritional support during the extended period of gestation, due to the noninvasive nature of epitheliochorial placentation (Jainudeen and Hafez, 1987). It has been demonstrated that uterine secretions are taken up by the chorion through areolae located in apposition to the mouths of uterine glands (Friess et al., 1981).

Uterine luminal fluids contain inorganic and organic molecules (glucose, fructose, riboflavin, ascorbic acid, sodium, potassium, and calcium), amino acids, hormones, and proteins (Roberts and Bazer, 1988; Simmen and Simmen, 1990)). The proteins in uterine secretions serve as enzymes, transporters, and regulators, which are involved in the modulation of the physiological processes essential for embryonic and uterine development (Roberts and Bazer, 1988; Simmen and Simmen, 1990).

Enzymes

Several enzymes found in porcine uterine secretions include acid phosphatase or uteroferrin (Zavy et al., 1984) for iron transport, leucine aminopeptidase (Roberts et al., 1976; Zavy et al., 1984) for regulation of endo-/exocytosis, lysozyme (Roberts et al., 1976) presumed for antibacterial activity, glucose phosphate isomerase (Zavy et al., 1982) for placental conversion of glucose to fructose, and cathepsins (B, D, E) for tissue remodelling during implantation (Roberts et al., 1976). Aminoacyl-

peptidase, ß-N-acetylglucosaminidase, esterase, hyaluronidase, oxytocinase, and several glycosidases also have been found (Bazer et al., 1991a).

Plasminogen activator (PA). PAs, which are arginine-specific serine proteases, in uterine secretions cleave inactive plasminogen to active plasmin (Littlefield, 1991). Two immunologically distinct PAs have been identified, namely urokinase-type PA (uPA) and tissue-type PA (tPA). In human uterus, tPA is the dominant PA in the myometrium, whereas both uPA and tPA are present in the endometrium. Both enzymes are similarly regulated in the uterus by steroids: activities are stimulated by estrogens and inhibited by progesterone. It has been reported that uPA, not tPA, is the PA type expressed by invasive trophoblast cells of the implanting mouse embryo (Sappino et al., 1989). In the pig, PA is secreted by blastocysts (Mullins et al., 1980); however, PA is not detected in uterine flushings of pregnant pigs after Day 11 (Fazleabas et al., 1983). The presence of protease inhibitors in uterine secretions (Mullins et al., 1980; Fazleabas et al., 1982; Farmer et al., 1989) suggests their inhibitory roles in the regulation of the invasive potential of pig trophoblast (Samuel and Perry, 1972; Roberts and Bazer, 1988). Epidermal growth factor induction of PA inhibitor in human hepatoma cells and PA mRNA and proteins in human HeLa cells (Lucore et al., 1988; Medcalf and Schleuning, 1991) indicates a possible regulation of PA by peptide growth factors in the uterus.

Plasmin/trypsin inhibitor (PI). PI (M_r of 14,000), a member of Kunitz class of protease inhibitor (Laskowski and Kato, 1980), is found in porcine uterine secretions during the estrous cycle (Mullins et al., 1980) and early pregnancy (Fazleabas et al., 1982). This protein exhibits amino acid sequence homology with aprotinin, a bovine pancreatic trypsin inhibitor (Fritz and Wunderer, 1983). Fazleabas et al. (1982) observed that progesterone, synergistically with estrogens, regulates the endometrial synthesis of PI. Around Days 10 to 16 of pregnancy, pig conceptus-derived estrogens are thought to stimulate secretion of endometrial PI into the lumen (Fazleabas et al., 1983). Since pig blastocysts secrete PA, PI is thought to regulate the activity of PA within the uterine lumen to maintain the integrity of the uterine epithelium in the face of the invasive potential of the trophoblast during early pregnancy.

Antileukoproteinase (ALP). An ALP, a lysosomal serine protease inhibitor, has been identified in pig endometrium (Farmer et al., 1989). Porcine ALP (pALP) (M_r of 14,000, pI of 8.2) (Simmen et al., 1992) exhibits 70% amino acid sequence homology to human ALP (Seemuller et al., 1986; Farmer et al., 1989). Porcine ALP protein was detected in endometrium but not in placenta of early pregnant (Day 30) pigs, and immunolocalized in glandular and surface epithelium but not in endometrial stroma (Simmen et al., 1991, 1992). Uterine pALP mRNA levels are low at early (Day 12), but high at mid- and late- (Days 45-110) pregnancy (Farmer et al., 1989), a pattern which is similar to that for uteroferrin mRNA expression (Simmen et al., 1988b). The presence of ALP mRNA was also detected in equine

endometrium (Simmen et al., 1991). Expression in horse endometrium is different from that in pig endometrium: low levels at early-, high levels at mid-, and low levels at late-pregnancy. The uterine expression of pALP mRNA is regulated by steroids. Farmer et al. (1990) demonstrated that ALP mRNA synthesis is regulated mainly by estrogen, but that prolonged administration of progesterone could stimulate uterine ALP expression in prepubertal gilts. However, either steroid was without this effect in ovariectomized gilts; whereas the presence of conceptuses stimulated endometrial ALP mRNA expression (Simmen et al., 1991).

The exact functions of ALP during pregnancy are not known, but the increased expression of its mRNA transcript in uterine horns containing conceptuses suggests its possible involvement during implantation and placentation. Other possible functions of endometrial ALP include maintenance of the integrity of the placental membrane, modulation of protease activity to prevent premature rupture of fetal membranes, and prevention of invasive implantation and protein degradation (Simmen and Simmen, 1990; Farmer et al., 1990; Simmen et al., 1992).

Transport Proteins

Uteroferrin (Uf). Uf is the most abundant (as much as 15% of the total) and best characterized endometrial secretory protein (Roberts et al., 1986; Simmen et al., 1988b). Uf is synthesized as a precursor protein (M_r of 31,000), is secreted by glandular epithelium as a mature glycoprotein (M_r of 35,000, pI of 9.7), and detected in uterine secretions as early as Day 12 of pregnancy. The Uf chromosomal gene has

been isolated and characterized (Srinivas and Simmen, 1989) and its complete mRNA sequence (cDNA) reported (Simmen et al., 1989b). In the pig, the uterine expression of Uf protein and its mRNA are differentially regulated. Synthesis and secretion of Uf protein is regulated mainly by progesterone and synergistically by low concentrations of estrogen (Knight et al., 1974; Basha et al., 1980). The ontogeny of Uf protein expression is characterized by low levels at early (Day 12), high levels at mid (Days 60-75), and low levels at late (Days 75-105) pregnancy, whereas increased levels of Uf mRNA at mid-pregnancy are maintained until term (Simmen et al., 1988b). The similar patterns of Uf protein production and uterine secretory activity (Basha et al., 1979) throughout pregnancy suggest a correlation of Uf protein production with plasma steroid hormone levels. Both estrogen and progesterone stimulated secretion of Uf into the uterine lumen of immature and mature, ovariectomized gilts (Simmen et al., 1988b, 1991). Fliss et al. (1991b) transfected a Uf gene promoter reporter gene construct into rabbit endometrial cells to examine hormonal and tissue-specific regulation. An estrogen (E)/prolactin (PRL)-dependent response to progesterone by the Uf gene promoter was observed. This observation further led to the identification of nucleotide sequences in the 5′ flanking region of the Uf gene (Fliss et al., 1991a), which is similar to the consensus sequence of progesterone responsive elements (Beato, 1989). A similar E/PRL-dependent induction of Uf mRNA and protein in uterine endometrium (Fliss et al., 1991b) and Uf protein in uterine flushings (Young et al., 1990) from ovariectomized gilts was also observed.

Uf has acid phosphatase activity (Schlosnagle et al., 1974) and exhibits 80% homology in amino acid and nucleotide sequence with human placental acid phosphatase (Ketcham et al., 1989). Cow uterine secretions also contain an acid phosphatase which likely represents bovine Uf (Ketcham et al., 1985) and a Uf-like protein and mRNA were identified in equine uterine secretions and endometrium, respectively (McDowell et al., 1982; Simmen et al., 1991). However, the primary function of Uf during pregnancy is believed to be transplacental iron-transport (two irons per molecule) to fetuses (Roberts et al., 1986). Uf is sequestered by the areolae of the chorion, deposited in the umbilical vein, and transported to the fetal liver where it binds to receptors on the reticuloendothelial cells and is endocytosed via coated pits (Renegar et al., 1982). The high mannose carbohydrate portion of Uf serves as a part of the recognition marker for receptor binding (Saunders et al., 1985). Uf is then directed to lysosomes where it gives up its iron to ferritin which is subsequently transferred to erythroblasts for fetal hemoglobin synthesis by a process termed ropheocytosis. Uf not bound by the liver is cleared through the kidney into the fetal bladder and then, via the urachus, into the allantoic sac, a site for Uf degradation and the site for iron storage and exchange (Renegar et al., 1982; Buhi et al., 1983). In the allantois, Uf can transfer its iron to transferrin which is taken up by the allantoic epithelium and enters the fetal circulation where it can provide iron for hematopoiesis and meet other metabolic demands for iron (Buhi et al., 1983).

Uf has recently been shown to be a hematopoietic growth factor, possessing granulocyte - erythrocyte - monocyte / macrophage - megakaryocyte colony-forming unit (CFU-GEMN) activity in vitro (Bazer et al., 1991c).

Retinol binding proteins (RBPs). RBPs (M_r of 19,000-22,000, pI of 5.6-6.5) transport vitamin A which exists as a lipid alcohol retinol. Besides the liver as a major synthesis site for plasma RBP, RBPs are also secreted by endometrial epithelium under the influence of progesterone during the estrous cycle (Adams et al., 1981) and by pig conceptuses during Days 10-15 of gestation (Harney et al., 1990). Immunoreactive RBPs were detected in trophectoderm and in the yolk sac of conceptuses and both surface and glandular endometrial epithelium. They exhibit more than 90% amino acid sequence homology with human and rabbit RBPs, although uterine RBP differs from those in its relative affinity for retinol (Adams et al., 1981). The presence of RBP mRNA transcripts was demonstrated in Day 11 pig embryos (Trout et al., 1991). In addition, the secretion of RBPs by conceptuses of sheep (Day 15) and cow (Day 19) as well as pig was demonstrated by Western blot analysis of conceptus culture media (Trout et al., 1991). Bovine and ovine placenta also have been shown to secrete RBPs (Liu et al., 1990, 1992).

RBPs are likely to be involved in the establishment and maintenance of pregnancy based upon their modulation of transport of retinoids and the subsequent effects of these molecules on gene transcription, cellular proliferation and differentiation, steroidogenesis, epithelial cell integrity and function, hematopoiesis

and immune cell function, and interferon production from conceptuses (Harney et al., 1990).

Regulatory Proteins

Immunoregulatory factors. There are immunosuppressive factors present in uterine secretions which possibly suppress maternal lymphocyte responses. These include high M_r (>660,000) glycoproteins secreted from preimplantation ovine and porcine conceptuses (Murray et al., 1987) and the ovine uterine milk proteins (UTMPs) (Hansen et al., 1987). Ovine UTMPs consist of two basic glycoproteins of M_r 57,000 and 59,000 that are members of the serine protease inhibitor family (Ing and Roberts, 1989). These proteins are abundant in uterine secretions obtained from the ligated uterine horn of unilaterally pregnant ewes at Days 125-140 of gestation. UTMPs are secreted from the surface and glandular endometrial epithelium, a process which is induced by progesterone (Moffatt et al., 1987). Like uteroferrin, UTMPs have the lysosomal recognition marker, mannose-6-phosphate (Hansen et al., 1987). Uterine secretions of late pregnant (Days 200-270) cows also contain high M_r basic and acidic immunosuppressive proteins, the most active being the basic protein with a M_r of $>4x10^6$ (Segerson and Bazer, 1989). The physiological functions of these proteins are unknown, but their inhibition of lymphocyte blastogenesis in vitro has been demonstrated (Hansen et al., 1987). Therefore, it was suggested that uterine-derived UTMPs may serve in an immunoregulatory capacity by altering maternal, cell-mediated immune responses to protect developing conceptuses.

However, immunostimulatory factors (M_r of 29,000 and 14,000) are also present in the uterine secretions from the pregnant pigs (Segerson et al., 1991), which contrasts with the previous finding of immunosuppressive low M_r (15,000) uterine specific acidic proteins (Murray et al., 1978).

Local regulatory factors. $PGF_2\alpha$ and PGE_2 serve as local regulators of the intrauterine environment (Lewis and Waterman, 1983) by stimulating water and electrolyte transport, vasodilation, and capillary permeability that may facilitate implantation.

Peptide growth factors. Many growth factors including the insulin-like growth factor-I and -II, epidermal growth factor, acidic and basic fibroblast growth factors, transforming growth factors-α and -ß, colony-stimulating factor-1, and other as yet uncharacterized mitogens have been identified in the uterine microenvironment. These growth factors are thought to be involved in the regulation of conceptus and uterine development.

Uterine-Derived Peptide Growth Factors

Peptide growth factors are a group of peptide regulatory molecules with certain characteristics: (a) low M_r (usually less than 80,000); (b) mostly glycosylated proteins; (c) local production in various tissue types; (d) specific high affinity cell-surface

receptors; (e) autocrine, paracrine, and/or endocrine modes of action; and (f) ability to affect cellular proliferation and/or differentiation. Therefore, growth factors exert their mitogenic effect by interaction with specific cell surface receptors on responsive cells. Many growth factors form families of structurally related molecules that bind to common receptors. The binding of a growth factor to its receptor elicits a cascade of events, including protein phosphorylation, inositol-lipid breakdown, ion fluxes, and changes in gene expression. Several growth factors and their receptors have been identified and characterized in the uterine environment (Table 1-2).

Table 1-2. Uterine-derived peptide growth factors.

Growth Factors*	M_r ($x10^{-3}$)	Presence of Receptors	Steroidal Regulation**	Species
IGF-I	7.6	embryo/uterus	E/P	rodents, pig, sheep, cow, human
IGF-II	7.5	embryo/uterus	?	
EGF	6	embryo/uterus	E	rodents
TGF-α	7.5	embryo/uterus	E	rodents, cow
TGF-β	25	?	?	rodents, cow, human
acidic FGF	16	embryo/uterus	?	pig, cow, human, rodents
basic FGF	17	embryo/uterus	E	
PDGF	28-32	embryo/uterus	?	mouse, human
CSF-1	70-90	embryo/uterus	E/P	mouse, human

* IGF (insulin-like growth factor)-I and -II, EGF (epidermal growth factor), TGF (transforming growth factor)-α and -β, FGF (fibroblast growth factor), PDGF (platelet-derived growth factor), and CSF (colony-stimulating factor)-1.

** E (estrogens) and P (progesterone).

Insulin-like Growth Factors (IGFs)

IGF-I. IGF-I, also known as somatomedin C, is a basic polypeptide of 70 amino acids (AA) which is structurally related to proinsulin and more distantly to relaxin (Blundell and Humbel, 1980). IGF-I is ubiquitously produced by a variety of cell types and tissues, binds to cell surface receptors to mediate cell division and differentiation, and functions in vivo via autocrine, paracrine, and endocrine routes (Zapf and Froesch, 1986).

IGF-I is implicated as a local mediator of estrogen action during uterine growth (estromedin) (Sirbasku, 1980) primarily from the stimulatory effects of estrogen on IGF-I mRNA and protein biosynthesis. The estrogen-induced growth factor, termed uterine-derived growth factor, in pregnant sheep and porcine uterus was identified as a truncated form of IGF-I (Ikeda and Sirbasku, 1984; Ogasawara et al., 1989). Murphy et al. (1987b) observed increased expression of IGF-I and its mRNA transcripts in the uterus of ovariectomized, immature rats after estrogen administration. Mature, cyclic pigs exhibited temporal changes in uterine luminal fluid (ULF) IGF-I concentrations with peak levels on Days 10-12 of the estrous cycle (Simmen et al., 1989a). Also, increased levels of IGF-I mRNA were observed in uterine tissues of immature and mature ovariectomized gilts after estradiol or progesterone treatments (Simmen et al., 1990). Similarly, IGF-I mRNA expression was stimulated by estrogens and/or progesterone in the ovariectomized adult mouse uterus (Kapur et al., 1992). Collectively, these results suggest that IGF-I functions

as a local regulator of uterine growth and differentiation, particularly during periods of high circulating steroid concentrations.

In pregnant animals, expression of uterine IGF-I is closely associated with early embryonic development. Porcine IGF-I (pIGF-I) cDNA clones were isolated and the primary amino acid sequence of pIGF-I reported (Tavakkol et al., 1988). Blot-hybridization of uterine endometrial RNAs with cloned cDNA demonstrated highest levels of uterine IGF-I mRNAs during early pregnancy (Days 10-12), with low to undetectable levels at later stages of pregnancy. Pig embryos exhibit dramatic developmental and morphological changes during Days 10-12, suggesting a relationship of uterine IGF-I and conceptus development in the pig. To establish a basis for understanding the physiological roles of IGF-I during early pregnancy in pigs, Letcher et al. (1989a) analyzed the ontogeny of IGF-I mRNA expression in uterine endometrial and conceptus tissues, and the levels of IGF-I in ULF from early pregnant pigs. IGF-I content in endometrium was unchanged during this period, whereas the highest levels of IGF-I mRNA in endometrium were on Day 12 and then declined thereafter. Similarly, IGF-I content in ULF was low on Day 8, increased to maximal levels on Day 12, and then declined by Day 14. Content of IGF-I in conceptus tissues also exhibited a peak on Days 10-12. IGF-I content in ULFs from gravid uterine horns exceeded that for ULFs from nongravid horns (Simmen et al., 1990). Although a possibility for species-specific expression of uterine IGF-I cannot be excluded, these results support the hypothesis that IGF-I acts as a mediator of uterine and conceptus growth and differentiation during the estrous cycle and early

pregnancy. This hypothesis is supported by other findings as well. Heath and Rees (1985) suggested that IGFs are survival factors for embryonic cells. Mercola and Stiles (1988) demonstrated that the production of IGFs is generally enhanced upon cellular differentiation. After cellular differentiation, embryonal carcinoma cells synthesize and secrete polypeptide growth factors, suggesting the involvement of such factors during early embryonic development. Contrary to the above, IGF-I did not influence mouse embryonic development in vitro although insulin was stimulatory (Paria and Dey, 1990). However, the fact that exogenous IGF-I, but not insulin, stimulated the cytochrome aromatase P450 activity of porcine conceptuses in culture (Hofig et al., 1991b) and that exogenous IGF-I stimulated protein synthesis and secretion by pig embryonic discs in vitro (Estrada et al., 1991) suggests an important role for IGF-I in porcine embryonic development. In related studies, Geisert et al. (1991) identified IGF-I and IGF-II in bovine uterine luminal fluids during the estrous cycle and early pregnancy. Endometrial expression levels of IGF-I mRNA were unchanged during the estrous cycle and early stages of pregnancy, thus the differences in ULF IGF-I content may represent enhanced secretion of IGF-I stimulated by the surge of follicular estrogens. In contrast, the levels of IGF-I in ovine uterine flushings were unchanged during the estrous cycle and early pregnancy (Ko et al., 1991). Presence of IGF-I mRNA in uterine tissues was confirmed in the rat (Murphy et al., 1987a) and human (Hoppener et al., 1988), and IGF-I mRNA in the mouse uterus showed the highest levels around the initiation of implantation (Days 4-6) (Kapur et al., 1992). IGF-I mRNAs were localized in the stromal and

smooth muscle layers of the rat uterus (Croze et al., 1990). However, mouse IGF-I gene is expressed in a cell type-specific fashion (Kapur et al., 1992). In situ hybridization and immunohistochemical studies demonstrated spatial changes in the levels of both IGF-I mRNA and protein from the glandular and luminal epithelial cells, to the stromal cells, and to the decidual cells as pregnancy proceeds.

Type I IGF receptors. The Type I IGF receptor (IGF-I-R) is a heterotetramer of $\alpha_2\beta_2$ subunits, which is structurally related to the insulin receptor, has intrinsic tyrosine kinase activity, and binds IGF-I, IGF-II, and insulin, albeit with differing affinities (Sara and Hall, 1990). By competitive binding and cross-linking studies, IGF-I-Rs were found in the myometrium of immature rat uterus (Ghahary and Murphy, 1989), and in endometrium and myometrium of the porcine uterus (Hofig et al., 1991a). Also, trophoblast cells from preimplantation pig blastocysts display binding sites for IGF-I (Corps et al., 1990). IGF-I stimulated DNA synthesis in porcine endometrial stromal cells (Simmen et al., 1988c), but not in mouse uterine epithelial cells in vitro (Tomooka et al., 1986). Uterine IGF-I-R appears not to be regulated by steroids. Similar to the observed lack of correlation of stage of the estrous cycle with human myometrial IGF-I-R numbers (Tommola et al., 1989) and porcine uterine IGF-I-R numbers (Hofig et al., 1991a), the abundance levels of IGF-I-R mRNAs in porcine endometrium were unchanged during the estrous cycle and throughout pregnancy (Simmen et al., 1992). These results are consistent with the

observation that the low abundance level of IGF-I-R mRNA was not changed during the peri-implantation period in the mouse (Kapur et al., 1992).

IGF-II. IGF-II, a 67 AA and slightly acidic peptide, exhibits ~70% homology with IGF-I and is considered to be less growth hormone-dependent than IGF-I. IGF-II is found at high concentrations in fetal and neonatal sera of rodents and transcription of its gene is strongly down-regulated postnatally. This peptide growth factor classically has been considered a fetal growth factor (Rotwein, 1991). The observed high levels of IGF-II mRNAs in the cyclic rat uterus (Murphy et al, 1987a), human decidua (Ohlsson et al., 1989), pig uterus at mid-pregnancy (Simmen and Simmen, 1990; Simmen et al., 1992), and human fetal uterus (Yeh et al., 1991) suggest IGF-II-specific function(s) in this tissue. Administration of estradiol-17β led to an induction of IGF-II mRNA levels in the uterus of ovariectomized, hypophysectomized rats (Murphy et al., 1987b). However, the opposite finding was also reported, namely that estrogen did not alter IGF-II mRNA steady-state levels in an adult ovariectomized or immature rat uterus (Nordstedt et al., 1989). In pigs, low levels of IGF-II mRNAs are characteristic of the endometrium during early pregnancy. High levels of mRNAs are found during midgestation, where expression levels in endometrial epithelial cells exceeds that in endometrial stromal cells (Simmen et al., 1992). In immature pigs, uterine IGF-II mRNA levels are low and unaffected by estrogen or progesterone (Simmen et al., 1990). Cyclic and pregnant cows also express IGF-II mRNAs in endometrial tissues. The level of endometrial

IGF-II mRNA expression in pregnant cows is greater than that in cyclic cows, suggesting a stimulatory effect of presence of conceptuses (Geisert et al., 1991). IGF-II content in ovine ULFs increased during trophoblast expansion, in which filamentous conceptuses in part provide a source of IGF-II (Ko et al., 1991). The coexpression of IGF-II mRNA and Type I and Type II IGF receptor mRNAs in human trophoblast have been reported (Osborne et al., 1989). IGF-II is an inhibitor of aromatase activity in human placental cytotrophoblasts (Nestler, 1990), indicating a possible regulatory role for IGF-II in placental steroidogenesis.

Type II IGF receptors. The Type II IGF receptor (IGF-II-R) is a monomeric protein (M_r of 250,000) identical to the cation-independent mannose-6-phosphate receptor (MacDonald et al., 1988). This receptor does not exhibit tyrosine kinase activity and has no apparent affinity for insulin (Humble, 1990). IGF-II-Rs are coexpressed with IGF-II in rat embryos, suggesting local actions of this growth factor during embryonic development (Senior et al., 1990). The IGF-II-R gene in breast cancer cells was down-regulated by estrogen treatment (Mathieu et al., 1991); however, the regulation by steroids of IGF-II-R expression in the uterus remains to be elucidated.

IGF binding proteins. Circulating IGFs are invariably associated with binding proteins (IGFBPs) as an IGF-IGFBP complex (Rosenfeld et al., 1990; Sara and Hall, 1990). IGFBPs modulate (stimulate or inhibit) bioactivities of the IGFs (Elgin et al.,

1987; Ritvos et al., 1988a; Blum et al., 1989). Recent interest in the IGFBPs has resulted in the identification and characterization of at least six distinct, though structurally-related, IGFBPs (Rosenfeld et al., 1990; Shimasaki et al., 1991). Among the uterine-expressed IGFBPs, IGFBP-1, which is identical to placental protein 12 (Rutanen et al., 1986) and pregnancy-associated endometrial α_1-globulin (α_1-PEG) (Waites et al., 1988), is best characterized. This IGFBP is a growth hormone-independent, nonglycosylated protein (M_r of 29,000-32,000). The expression of IGFBP-1 is inhibited by estrogen (Murphy and Ghahary, 1990) and stimulated by progesterone (Rutanen et al., 1987). IGFBP-1 is a major secretory protein of the endometrium and its mRNA is expressed in human endometrial stromal cells (Julkunen et al., 1990), in rat endometrial luminal epithelium and stroma (Murphy and Ghahary, 1990) and in rat decidual tissue (Croze et al., 1990). A presumptive IGFBP-1 was shown to be synthesized by rabbit endometrial epithelial cells in vitro (Ko et al., 1992). Use of a monoclonal antibody to α_1-PEG localized this protein only in the luminal epithelium of early pregnant sheep uterus (Waites et al., 1990). IGFBP-1 staining was maximal at Day 14 and absent by Day 16. Since attachment of trophoblast to uterine epithelium in sheep is initiated at Day 14, which is also when high levels of IGFs occur in ULF (Ko et al., 1991) and when maternal recognition of pregnancy occurs (Godkin et al., 1984b), results suggest a role for IGFBP-1 and IGFs in preimplantation sheep conceptus development. Elucidation of the temporal relationships among the endometrial-secreted IGFs and IGFBP, and

the conceptus-secreted proteins are necessary to understand their physiological roles and possible synergistic interactions during early pregnancy.

Other subclasses of IGFBPs, IGFBP-2 and -3, are also expressed in the uterus; however, uterine functions of these proteins are poorly understood (Margot et al., 1989; Giudice et al., 1991; Geisert et al., 1991; Simmen et al., 1992). Complementary DNAs encoding IGFBP-2 and IGFBP-3 have been cloned and sequenced (Margot et al., 1989; Wood et al., 1989). In pigs, uterine IGFBP-2 mRNA expression is unique (Simmen et al., 1992) since it is expressed strictly in endometrium and undetectable in myometrium and placenta. Also, its ontogeny of expression contrasts with that for IGF-I mRNAs (Letcher et al., 1989a), highest during estrus and lowest around Day 10 of gestation. IGFBP-2 mRNA expression is also regulated by steroids in cow endometrium (Geisert et al., 1991). The increased expression of IGFBP-2 mRNA has been demonstrated during the luteal phase of the estrous cycle and during early pregnancy (Days 10-18), but differences in IGFBP-2 mRNA expression between cyclic and pregnant cows were not detected. The expression of uterine IGFBP-2 mRNA regulated by estrogens in the pig and by progesterone in the cow which contrasts with steroid regulation in other species. In the rat, IGFBP-2 mRNA abundance is greater in the placenta than in the uterus (Margot et al., 1989). In the human, endometrial IGFBP-2 and -3 mRNAs are expressed in the endometrium during menstrual cycle (Giudice et al., 1991). Proteins and mRNAs encoding IGFBP-2 and, to a lesser extent, IGFBP-3 are more abundant during the secretory (high estrogen and progesterone) than proliferative (high estrogen) phases of the

human menstrual cycle, indicating a progesterone-dependence and synergism with estrogen. Uterine IGFBP-3 mRNAs were also detected in adult rat, whose levels were comparable to the hepatic levels; however, levels of uterine IGFBP-3 mRNA were not correlated with those of IGF-I mRNA (Albiston and Herington, 1992).

Epidermal Growth Factor (EGF)

EGF is a single chain, heat-stable, acidic polypeptide (53 AA, M_r of 6,000) that has strong mitogenic activity for various cell types both in vivo and in vitro (Fisher and Lakshmanan, 1990). EGF was originally isolated from mouse submaxillary glands (Cohen, 1962) and subsequently from human urine (Gregory, 1975). Mature EGF is derived by proteolysis of the much larger preproEGF (1207 AA in human and 1217 AA in mouse) and the mature peptide is less structurally conserved among species than are the IGFs (Fisher and Lakshmanan, 1990).

Ample evidence indicates that uterine EGF synthesis is regulated by estrogen in rodents. Imai (1982) demonstrated the presence of an EGF-like factor and its dependence on estrogen in rat uterine luminal fluids. Simmen et al. (1988c) observed the presence of EGF or EGF-related molecules in unfractionated uterine secretions from pregnant gilts. The presence of EGF mRNAs and immunoreactive protein in immature mouse uterus and their stimulation by estrogens has been reported (Gonzalez et al., 1984; DiAugustine et al., 1988). EGF mRNA has been detected in porcine endometrium and found to be expressed at constant levels throughout gestation, but not increased by steroids in immature gilts (Tan Y, Simmen

RCM, Simmen FA, unpublished observations). The enhanced expression of EGF in mouse uterine epithelium by estrogen (Huet-Hudson et al., 1990) coupled with the estrogen-like effects of EGF in mouse uterine cell growth (Nelson et al., 1991) implicate EGF as a paracrine mediator of estrogen effects in the rodent uterus.

EGF is stimulatory to in vitro growth of rabbit endometrial cells (Gerschenson et al., 1979), immature mouse uterine luminal epithelial cells (Tomooka et al., 1986), fetal guinea-pig uterine cells (Sumida and Pasqualini, 1989), and rabbit endometrial epithelial cells (Ko et al., 1992). EGF may also regulate myometrial contractile activity (Gardner et al., 1987), implying a possible role in parturition.

The EGF receptor (EGF-R) is a single-chain glycosylated polypeptide (1186 AA, M_r of 170,000) that exhibits tyrosine kinase activity and sequence-relatedness to the v-erb-B oncogene product (Downward et al., 1984). EGF-Rs are expressed by mouse uterine epithelial cells (Tomooka et al., 1986; Brown et al., 1989), rat uterine tissues (Mukku and Stancel, 1985), all uterine cell types of the immature rat (Lin et al., 1988), and human endometrial and myometrial tissues (Sheets et al., 1985; Tommola et al., 1989). Biosynthesis of EGF-Rs appears to be under the control of estrogen. For example, estrogen increases the levels of EGF-R and EGF-R mRNAs in immature (Mukku and Stancel, 1985; Lingham et al., 1988) and mature (Gardner et al., 1989) rat uterus. However, total uterine EGF binding was unchanged during the menstrual cycle in women (Hofmann et al., 1984; Chegini et al., 1986). Therefore, the uterus is implicated as a target tissue site for EGF action in a number of species.

The expression of EGF and its receptor during early stages of embryogenesis is well-characterized. Along with the observed increases in EGF binding to rat (Chakraborty et al., 1988) and mouse (Brown et al., 1989) uterine membranes and the increased DNA and protein synthesis of pig (Corps et al., 1990) and mouse (Wood et al., 1989) embryos in vitro during the peri-implantation period, EGF-Rs have been detected on preimplantation trophoblasts from rabbit (Hofmann and Anderson, 1990), pig (Letcher et al., 1989b; Corps et al., 1990) and human (Hofmann et al., 1991), and mouse embryos at the eight-cell stage (Paria and Dey, 1990) or at compaction (Wood et al., 1989). Paria et al. (1991) reported that EGF receptors in mouse embryos are functional with respect to tyrosine phosphorylation and signal transduction.

Although EGF is produced from many cell types and its receptors were identified on embryonic cells, the principal site of EGF production during embryonic development is probably the uterus. Popliker et al. (1987) screened mouse embryos and neonates for EGF mRNA in tissues, but no detectable EGF mRNAs were found. After injection of ^{125}I-EGF to pregnant mice either systemically or directly into the uterine arteries, high levels of [^{125}I]EGF were found in the uterus and the placenta, indicating that maternal EGF can reach embryos and fetuses. EGF-Rs are expressed in placenta and EGF may affect differentiation of the trophoblast (Maruo and Mochizuki, 1987). Several studies have shown low levels of EGF and EGF precursor mRNA in fetal mouse tissues and higher levels of EGF receptor-binding activity than EGF immunoreactive material (Fisher and Lakshmanan, 1990). Because of cross-

reactivity of TGF-α to EGF-R, it has been proposed that the ligand for the fetal EGF receptor is TGF-α. In this regard, identification of TGF-α in preimplantation mouse embryos (Rappolee et al., 1988) and decidual tissues (Han et al., 1987) suggests a role for TGF-α in the growth and differentiation of early mammalian embryos via binding to EGF-Rs. Taken together, these results suggest that binding of EGF or other members of this family of ligands to EGF-Rs is associated with embryonic or uterine growth and differentiation.

Transforming Growth Factor-α (TGF-α)

TGF-α is a single-chain polypeptide of 50 AA (M_r of 7,500) derived from a 160 AA transmembrane precursor by protease cleavage. This protein exhibits 30% AA sequence homology with EGF (Marquardt et al., 1984) and binds to EGF receptors (Massague, 1983).

The presence of TGF-α in mouse embryos was verified (Twardzik, 1985). Han et al. (1987) examined the site of TGF-α mRNA expression during rat embryogenesis and found highest levels of TGF-α mRNA in maternal decidua and specifically in those endometrial regions closest to implantation sites. Tamada et al. (1991) detected the coexpressed TGF-α mRNA and protein in the luminal and glandular epithelia during preimplantation (Days 1-4) and in the decidua at the implantation site during the post-implantation (Days 5-8) period. Further, TGF-α mRNA was identified in embryos (Days 5-6). A recent finding indicating that TGF-α stimulates development of two-cell mouse embryos to the blastocyst stage (Paria and Dey, 1990)

suggests a role for this peptide in early stages of preimplantation embryo development. Dore et al. (1991) observed the presence of TGF-α in endometrial epithelium and TGF-α-stimulated uterine growth in cyclic and pregnant cows. The presence of a potential estrogen responsive element(s) in the 5'-flanking region of the TGF-α gene (Saeki et al., 1991) suggests that the regulation of embryonic and uterine development during early pregnancy may be mediated in part by estrogen-dependent expression of TGF-α.

Heparin-binding Growth Factors (HBGFs)

The high affinity of these protein factors for heparin, specifically heparan sulfate, found on cell surfaces and in extracellular structures, such as basement membranes, give rise to the name for this family of growth factors. Two well-characterized HBGFs are acidic fibroblast growth factor (aFGF) (pI 5.6, 140 AA) a single-chain peptide synthesized in brain and other neural tissues, and basic FGF (bFGF) (pI 9.6, 146 AA) a peptide synthesized in pituitary, brain, adrenals, and ovary. Both FGFs were purified to homogeneity, sequenced, and their primary sequences found to exhibit 55% sequence identity. Both are multifunctional peptides implicated in cellular growth and differentiation, cell attachment, angiogenesis, and embryonic development (Gospodarowicz et al., 1987; Brigstock, 1991). FGF receptors (FGF-R) that exhibit tyrosine kinase activity and that bind both aFGF and bFGF with differing affinities have been identified (Gospodarowicz et al., 1987).

The mechanism of FGFs actions are complex and not well understood. Although high affinity cell surface receptors for FGFs exist, the FGF protein precursors do not have the usual signal peptide sequence necessary for secretion from cells. This has led to suggestions of unusual pathways for FGF secretion and of intracrine modes of action (Logan, 1990). Extracellular matrix (ECM)-degrading enzymes, such as heparanase (Baird and Ling, 1987) or plasminogen activators (Mullins et al., 1980) can potentially release the ECM-bound FGFs into the surrounding environment (Folkman and Klagsbrun, 1987).

An acidic HBGF was isolated from extracts of pregnant and nonpregnant pig endometrium and myometrium and termed uterine-derived growth factor or UDGF (Brigstock et al., 1986). This protein stimulated DNA synthesis in Swiss mouse 3T3 cells in culture and had high affinity for heparin. By use of heparin-agarose affinity chromatography, two peaks of activity were obtained from extracts of Day 17 pregnant pig uterus and from ULFs corresponding to Days 15-19 of pregnancy (Brigstock et al., 1989): α-UDGF was shown to represent the acidic form of FGF and ß-UDGF the basic form of FGF. Porcine uterine extracts also contain a high M_r (27 kDa) form of bFGF (Brigstock et al., 1990). In addition, aFGF and bFGF-like mitogens were identified in human uterus (Brigstock, 1991) and multiple forms of HBGF was isolated from bovine uterus (Milner et al., 1989). The latter protein was more recently shown to have no functional homologies to FGFs (Bohlen et al., 1991).

FGFs are synthesized by vascular smooth muscle cells (Weich et al., 1990), epithelial cells (Gospodarowicz et al., 1987) and human endometrial adenocarcinoma

cell lines (Presta, 1988). The bFGF was localized in stromal and epithelial cells of mouse and guinea pig endometrium (Wordinger et al., 1990). Basic FGF can be considered an estromedin, since its synthesis was stimulated by estradiol-17β and inhibited by progesterone in human endometrial adenocarcinoma cell lines (Presta, 1988).

Roles for the FGFs in embryonic and uterine growth are only speculative. Since bFGF is angiogenic in vivo (Gospodarowicz et al., 1987), this protein is probably involved in uterine angiogenesis during the menstrual cycle or neovascularization during pregnancy, possibly in concert with interleukin-6 (Motro et al., 1990; Tabibzadeh et al., 1989). Heparin-binding endothelial mitogen(s) was identified in ovine endometrium during early gestation (Reynolds et al., 1990b), further supporting the involvement of HBGFs in placental and uterine angiogenesis. Basic FGF is an important mediator of mesoderm induction in early embryos (Slack et al., 1987; Seed et al., 1988; Gonzalez et al., 1990) and FGF-R were identified in chick embryos (Burrus and Olwin, 1989). Expression of aFGF as well as bFGF in the developing rat embryo was associated with cellular growth and differentiation (Fu et al., 1991). A role for bFGF in trophoblast proliferation was suggested from the observed stimulation by bFGF of proliferation of mouse placental cells (Hondermarck et al., 1990) and from the immunolocalization of bFGF in human placental cells (Cattini et al., 1991). Immature mouse uterine epithelial cells did not respond mitogenically in vitro to FGFs (Tomooka et al., 1986); however, human endometrial stromal cells in the presence of progesterone (Irwin et al., 1991) and rabbit endometrial epithelial

cells (Ko et al., 1992) were stimulated to proliferate by the FGFs. An identification of apically-exposed heparin/heparan sulfate binding sites by uterine epithelial cells (Wilson et al., 1990) further supports the involvement of FGFs in embryo implantation.

Hematopoietic Growth Factors

Their name was derived from the ability of such factors to stimulate the formation of colonies of neutrophilic granulocytes and monocyte-macrophages. Many cell types including fibroblasts, macrophages, endothelial cells, and T and B lymphocytes, can produce such factors in response to inductive stimuli (Pollard, 1990).

An early observation of high levels of a factor in pregnant mouse uterus and fetal tissues that stimulated growth of bone marrow cells (Bradley et al., 1971) led to the subsequent identification of colony-stimulating factor-1 (CSF-1). CSF-1, also called monocyte-macrophage colony stimulating factor (M-CSF), is a homodimeric glycoprotein (M_r of 90,000) essential for proliferation, differentiation, and survival of mononuclear phagocytic cells (Stanley, 1986). Since the conceptus is a semi-allograft, a role for immunoregulatory proteins, such as hematopoietic growth factors, in the uterus is suggested. Bartocci et al. (1986) showed that during pregnancy there is an induction (1,000-fold increase) in murine uterine CSF-1 content. This induction is possibly regulated by chorionic gonadotropin (CG) and steroids since CG-mediated induction of CSF-1 was diminished in ovariectomized mice (Bartocci et al., 1986).

Pollard et al. (1987) later showed that the levels of uterine CSF-1 are regulated by the synergistic actions of 17β-estradiol and progesterone. CSF-1 mRNA was detected in pregnant mouse uterus, but not in the non-pregnant mouse uterus, by Northern blot (Pollard et al., 1987) and in situ hybridization procedures (Arceci et al., 1989). This mRNA expression was localized to the luminal and glandular epithelium throughout pregnancy. The presence, during early pregnancy, of CSF-1 and its receptor in human endometrium and placenta was reported, suggesting a role for CSF-1 in decidual function and placental growth via interactions with its receptor (Kauma et al., 1991). Unlike the situation in the mouse, however, CSF-1 mRNAs were found in endometrial tissues throughout the menstrual cycle.

The CSF-1 receptor is a glycoprotein (M_r of 165,000) with intrinsic tyrosine kinase activity and is encoded by the c-fms proto-oncogene (Sherr et al., 1985). Its mRNA transcripts were detected in mouse trophectoderm and endometrial stromal cells (Arceci et al., 1989; Regenstreif and Rossant, 1989), human syncytial trophoblast (Visvader and Verma, 1989) and uterine glandular epithelium (Pampfer et al., 1992), and bovine trophoblast (Beauchamp and Croy, 1991). Increased DNA synthesis in mouse placental cells in vitro upon addition of CSF-1 was observed (Athanassakis et al., 1987). These findings indicate that CSF-1 is likely to be a mediator of placental growth and development under the control of steroidal and other hormones.

Another putative hematopoietic growth factor of the uterus is uteroferrin (Uf), a progesterone-inducible pig endometrial protein (Roberts et al., 1986; Simmen et al., 1988b). Uf has colony-forming unit (CFU) activity for granulocyte-erythrocyte-

monocyte/macrophage-megakaryocyte cell lines. Uf possibly acts as a colony stimulating factor to regulate blood cell production and differentiation, possibly by binding to primitive stem cells, as well as serving in its capacity as an iron transport protein during pregnancy (Bazer et al., 1991c).

Platelet-derived Growth Factor (PDGF)

PDGF is a basic (pI 9.8-10) glycoprotein acting as a mitogen and a chemotactic agent for various cell types. PDGF consists of either homo- or heterodimers of A (M_r of 16,000) and B (M_r of 14,000) chains, which exhibit 60% amino acid sequence homology. These PDGF dimers are remarkably resistant to heat and chemical denaturants (Ross et al., 1986; Heldin and Westermark, 1991). The B-chain of PDGF is the product of the c-sis protooncogene (Waterfield et al., 1983).

PDGF initiates its biological effects by binding to its cell surface receptor which is a transmembrane glycoprotein (M_r of 170,000-180,000) with intrinsic protein tyrosine kinase activity (Deuel, 1987).

Mercola et al. (1988) showed that the A chain of PDGF is encoded by maternal mRNA stored within Xenopus eggs, implicating this growth factor in the very early stages of amphibian embryogenesis. PDGF A-chain mRNAs are synthesized in preimplantation mouse blastocysts (Rappolee et al., 1988), suggesting roles for PDGF in mammalian embryogenesis. High levels of PDGF-B gene (c-sis) mRNAs were found in human cytotrophoblast (Goustin et al., 1985). Mouse uterine epithelial cells showed no mitogenic response to PDGF (Tomooka et al., 1986); however, PDGF

receptors were identified in porcine uterus, preferentially associated with endometrial stromal cells (Rönnstrand et al., 1987). The recent identification of PDGF in human myometrium, but not in endometrium, and its decreased expression after parturition suggest a specific role during uterine expansion via the stimulation of smooth muscle cell proliferation (Mendoza et al., 1990).

Transforming Growth Factor-β (TGF-β)

TGF-β1 (originally described as TGF-β) is a disulfide-linked homodimer (M_r of 25,000, 112 AA/monomer) synthesized in an inactive form that is subsequently activated by changes in pH, addition of dissociating agents, incubation with enzymes, or incubation with certain cell types (Sporn and Roberts, 1991; Miyazono and Heldin, 1991). TGF-β1 belongs to a large family of both closely and distantly related proteins. TGF-β1 and 2 have been purified from mammalian cells and tissues, whereas TGF-β3, 4 and 5 were cloned by low stringency hybridization from Chinese hamster, chicken and frog cDNA libraries, respectively (Miyazono and Heldin, 1991). TGF-β1, 2 and 3 share most of their biological activities. Distantly related members of TGF-β superfamily include mammalian inhibins and activins (Mason et al., 1985), Mullerian Duct Inhibiting Factor (Cate et al., 1986), and Xenopus Vg1 (Weeks and Melton, 1987). TGF-β is a multifunctional polypeptide which can affect cell proliferation and differentiation, synthesis of extracellular matrix components, and morphogenesis during development (Sporn and Roberts, 1991).

Uterine TGF-ß1 mRNAs are expressed by uterine epithelium and by decidua during periimplantation stages of mouse pregnancy (Tamada et al., 1990) and by human proliferative endometrium and all cell types in maternal-fetal interface throughout pregnancy, with the highest expression in term placenta (Kauma et al., 1990). Kauma et al. (1990) also observed the presence of TGF-ß protein in human uterine tissues throughout pregnancy, similar to the observation in human placenta by Frolik et al. (1983). Dore et al. (1991) immunolocalized the presence of TGF-ß1 in the uterine luminal epithelium of the early pregnant cow, providing additional support for its role in uterine development. Although TGF-ß inhibited proliferation of rat trophoblast cells (Hunt et al., 1989), it increased the rate of blastocyst formation in vitro (Paria and Dey, 1990). TGF-ß is considered a general inhibitor of epithelial cell proliferation (Edwards and Heath, 1991). In this regard, TGF-ß1 inhibited the growth of rabbit endometrial epithelial cells and antagonized the stimulatory activities of other growth factors in vitro (Ko et al., 1992). TGF-ßs may participate in neovascularization and immunoregulation during pregnancy (Massague et al., 1991). Also, a maternal mRNA (Vg1) encoding a TGF-ß-like protein was localized to the vegetal pole in Xenopus eggs (Weeks and Melton, 1987), suggesting the regulation of mesoderm formation by TGF-ß. Taken together, these results suggest that TGF-ß expression is temporally correlated with implantation, decidualization, placentation, and embryogenesis.

Three types of specific, high-affinity TGF-ß receptors are identified on various cell types (Segarini, 1991): glycosylated type I (M_r of 55,000-65,000) and type II (M_r

of 70,000-85,000), and high M_r proteoglycan type III (M_r of 200,000-280,000). The receptors and signal transduction mechanisms involved in the biological actions of TGF-ß have not been completely elucidated. Similarly, any observations regarding their expression in uterine and embryonic tissues have not been reported.

Tumor necrosis factor (TNF)-α protein is found in human placental and decidual tissues (Jaattela et al., 1988). This factor inhibited proliferation of rat trophoblast cells in vitro (Hunt et al., 1989); however, its exact physiological roles in reproductive and embryonic tissues have not yet characterized.

These factors have been speculated to serve as negative regulators of trophoblast invasion and of macrophage functions in the uterus during early pregnancy.

Other Growth-Promoting Factors

A mitogen for ovine trophoblast-derived cells and for rat L6 myoblasts was detected in ULFs of late-pregnant sheep (Bird et al., 1988). This factor of maternal-origin is greater than M_r of 30,000, has no affinity for heparin-agarose, does not show cell-type specificity, but is otherwise uncharacterized.

A distinct growth promoting factor, designated uterine luminal fluid mitogen (ULFM), was partially purified and characterized from pig ULFs (Simmen et al., 1988c). ULFM, present in ULF from both pregnant and nonpregnant pigs, has a Mr of 4,800, is acidic (pI 6.4), heat-stable, and relatively cell-type specific. ULFM appears to be a novel factor based upon several criteria. The partial purification and characterization of ULFM is reviewed further in Chapter 2.

Factors stimulatory to glucose transport and endometrial fibroblast proliferation were also identified in extracts of rat and rabbit uteri (Conrad-Kessel et al., 1988) and uterine cancer cells (Matsunami et al., 1989), respectively.

Summary

Results from embryo transfer experiments and biochemical and morphological analyses suggest that asynchronous development of embryos and uterus is a determinant of embryonic mortality. Although the embryonic factor(s) causing changes in the uterine environment during early pregnancy are not well understood, the period of maternal recognition of pregnancy appears to be a critical time for embryonic survival as well as for establishment and maintenance of pregnancy. During this period, estrogens or proteins from conceptuses acting via a luteostatic or antiluteolytic pathway are believed to be the signals required for maintenance of endometrial secretory activity. Although all of the uterine secretory products have not been characterized, several classes of proteins are thought to support conceptus development and/or uterine differentiation, more importantly of species with noninvasive types of placentation. Recent identification and characterization of several peptide growth factors derived from the conceptus and uterus have enhanced our understanding of their effects on reproductive functions, such as uterine development, embryogenesis, implantation, and placental development. However, further studies are required to understand the regulatory aspects of their actions on

embryos and uterus. Therefore, to elucidate the biochemical nature of uterine-derive peptide growth factors and their potential involvement in coordinate regulation of endometrial and conceptus development, studies regarding the identification, characterization, and biological roles during early pregnancy of uterine-derived peptide growth factors were conducted and are described in the following chapters.

CHAPTER 2
PARTIAL PURIFICATION AND INITIAL CHARACTERIZATION OF A UTERINE CELL MITOGEN IN PORCINE UTERINE LUMINAL FLUIDS

Introduction

Uterine luminal fluid (ULF) contains a complex array of molecules which originate from the circulation, conceptuses, and the uterus (Knight et al., 1973; Voss and Beato, 1977; Roberts and Bazer, 1988; Simmen and Simmen, 1990). The secretory patterns and biological activities of certain classes of molecules in ULF are under hormonal control. In particular, estrogens and progesterone have been shown to modulate synthesis and secretion of uterine proteins (Knight et al., 1973; Aitken, 1977; Surani, 1977; Geisert et al., 1982c; Kuivanen and DeSombre, 1985), uterine blood flow (Ford, 1989), uptake and transport of specific serum proteins by the uterus (Finlay et al., 1981), and the paracellular filtration of plasma components across the endometrium into the uterine lumen (McRae and Kennedy, 1979). Various classes of ULF proteins are thought to regulate embryo and fetal development, especially in species having noninvasive types of placentation. ULF proteins also appear to mediate uterine function and/or maternal-fetal interactions by serving as enzymes, transporter proteins, and growth mediators (Roberts and Bazer, 1988; Simmen and Simmen, 1990).

One regulatory class of uterine proteins are polypeptide growth factors. A number of peptide growth factors has been identified in and/or isolated from uterine tissues. These include insulin-like growth factors (IGFs) (Ogasawara et al., 1989; Simmen and Simmen, 1990; Murphy and Ghahary, 1990), epidermal growth factor (EGF) (Tabibzadeh, 1991), colony-stimulating factors (CSFs) (Pollard, 1990), transforming growth factors (TGFs) (Nickell et al., 1983; Tamada et al., 1990, 1991), platelet-derived growth factor (PDGF) (Mendoza et al., 1990), and fibroblast growth factors (FGFs) (Brigstock et al., 1989). However, reports of specific mitogenic factors in ULF are more limited (Flint, 1981; Leland et al., 1983; Bird et al., 1988).

Several questions concerning the regulation by growth factors of embryonic, fetal, and uterine development remain to be elucidated. These include identification of growth factors that are involved in the regulation of embryonic and fetal growth and uterine development, elucidation of the mechanisms by which growth factors are transported into the uterine lumen, elucidation of the control of growth factor action via the synergistic or antagonistic action of other growth factors or hormones, and elucidation of their mechanisms of actions in target tissues. The elucidation of these questions may eventually lead to the identification of factors which influence embryonic development during early pregnancy. In the present study, the mitogenic activity of ULF obtained from gilts at different physiological states was analyzed in an initial attempt to address these questions. The pig was chosen as the animal model for these studies since relatively large amounts of ULF proteins, uterine tissues, and embryos can be readily obtained during development. In this study, a

novel fibroblast mitogen, distinct from other known peptide growth factors, was identified, partially purified, and characterized.

Materials and Methods

Materials

All tissue culture media, serum, reagents, and other supplies were purchased from GIBCO (Grand Island, NY). Purified mouse EGF (mEGF, receptor grade) and rabbit polyclonal antiserum specific for mEGF were obtained from Collaborative Research (Bedford, MA). Recombinant human IGF-I was from Amgen Biologicals (Thousand Oaks, CA). The [^{3}H]thymidine (6.7 Ci/mmole) was from New England Nuclear (Boston, MA). Sephadex G-50 and G-200 resins were from Pharmacia (Piscataway, NJ) and DE-52 diethylaminoethyl (DEAE) cellulose was from Whatman Ltd. (England). The C_{18} μBondapak reverse-phase columns were from Waters (Milford, MA). All other reagents used were of analytical grade.

ULF Collection and Preparation

Gilts assigned to the pregnant group were mated 12 and 24 h after the onset of estrus (Day 0). Those gilts not allowed to mate were assigned to the cyclic group.

Anesthesia was induced with thiamylal sodium (1 gram/20 ml 0.9% NaCl) and maintained with 3-5% halothane. A midventral laparotomy was performed and the reproductive tract was removed. ULF was collected from both uterine horns of Days 10 and 12 pregnant and cyclic gilts by flushing each horn with 20 ml of 0.9% saline solution. Identification of embryos in the ULF confirmed pregnancy. The flushings

from both horns of two to three gilts were pooled, clarified by low-speed centrifugation (4,200 x g) at 4°C, and concentrated ~10-fold by ultrafiltration using an Amicon Y2 filter (M_r cut-off = 1,000) (Amicon Corp., Lexington, MA). Protein content of ULF was determined by the Bradford protein assay (Bio-Rad., Rockville Centre, NY) (Bradford, 1976) using bovine gamma globin as standard. The concentrated samples were stored at -20°C until later use.

Cell Culture

Mouse embryo-derived AKR-2B fibroblast cells were provided by Dr. H.L. Moses (Vanderbilt University, Nashville, TN). Madin-Darby canine kidney (MDCK) epithelial cells and human A431 epidermoid carcinoma cells were obtained from the American Tissue Type Culture Collection (ATTCC, Rockville, MD). All three cell lines were propagated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% antimycotic-antibiotic solution and 10% heat-inactivated calf serum (CS) for AKR-2B and A431 cells or fetal calf serum (FCS) for MDCK cells. Cells were grown in monolayers at 37°C in an atmosphere of 5% CO_2-air. Cells were subcultured using a solution (ATV) containing 0.14 mM NaCl, 5 mM KCl, 7 mM $NaHCO_3$, 1% dextrose, 0.05% trypsin, and 0.5 mM ethylenediaminetetraacetate (EDTA).

Primary cultures of stromal cells were established from uterine tissues of Day 12 pregnant pigs following published procedures (McCormack and Glasser, 1980), except that the trypsin concentration was increased to 0.25% and incubation with trypsin and DNase I (200 units/ml, bovine pancreatic, Sigma Chemical Co., St. Louis, MO)

carried out at 37°C for 30 min. The resulting cells were identified to be stromal based on their fibroblastic appearance under the light microscope. Cell viability was estimated by trypan blue exclusion and was typically more than 90%. The stromal cells were plated in DMEM containing 10% FCS and grown as described above. Mitogen assays utilized cells from three to six passages.

Mitogen Assay

Dispersed cells were seeded into 24-well plates (surface area of 1.77 cm^2/well) at a density of 5 x 10^4 cells/well and incubated until confluent monolayers were formed (72-96 h). The medium was aspirated and changed to 1.0 ml of DMEM containing 2% CS for A431 and AKR-2B or 2% FCS for uterine stromal and MDCK cells. The majority of the cells become nonproliferating in these media formulations after 48 h due to density-arrested growth inhibition and depletion of serum growth factors. Samples were filter-sterilized through 0.22 μm filters (Acrodisc, Gelman Sciences, Ann Arbor, MI). At this point, sterile test samples [10-200 μl with all the same final volumes adjusted with phosphate-buffered saline (PBS; 0.01 M $NaPO_4$, 0.15 M NaCl; pH 7.4)] were added to the wells (n=3) containing depleted medium and incubation was continued for an additional 20 h. The cells were then pulse-labeled with [^{3}H]thymidine (2 μCi/ml/well) for 4 h. Medium was removed and the cells washed with PBS, fixed in absolute methanol, rinsed in several changes of distilled water and 5% trichloroacetic acid (TCA), and solubilized in 0.3 ml of 0.3 M NaOH. Cell-associated [^{3}H]thymidine activity was then determined by liquid scintillation counting. For estimates of fold-purification, one unit of mitogenic

activity was arbitrarily defined as an amount of factor needed to stimulate half-maximal incorporation of [^{3}H]thymidine into DNA of density-arrested, quiescent AKR-2B cells.

Column Chromatography

For purification and/or estimation of M_rs of mitogenic factors, ULF samples were applied to Sephadex G-200 (45 x 2.5 cm), Sephadex G-50(c; coarse grade, 5 X 100 cm) or Sephadex G-50(f; fine grade, 50 x 2.5 cm) columns preequilibrated with PBS (pH 7.4). Proteins were eluted in PBS at 4°C with a flow rate of 20-45 ml/h. The individual fractions were tested for mitogenic activity and peak mitogenic fractions were pooled and concentrated by ultrafiltration for further purification. The columns were calibrated with M_r standards comprising a mixture of bovine thyroglobulin (670,000), bovine gamma globulin (158,000), chicken ovalbumin (44,000), horse myoglobin (17,000), bovine insulin (5,700), and vitamin B-12 (1,350). The M_r of mitogenic factor was estimated by a regression line from M_r standards.

Anion-exchange chromatography was performed on a DEAE cellulose column preequilibrated with 0.01 M Tris-HCl (pH 8.3) at 4°C. Proteins were loaded on the column and the column was washed with 0.01 M Tris-HCl (pH 8.3) to baseline absorbance at 280 nm. The column flow rate was ~30 ml/h. Bound proteins were then eluted with a linear gradient of 0-3 M NaCl in the same buffer. The fractions corresponding to each peak were pooled, dialyzed to desalt, and tested for the presence of mitogenic activity. Pools of peak mitogenic fractions were concentrated by ultrafiltration and used for further purification.

Mitogenically active fractions obtained from the above purification steps were pooled and concentrated by ultrafiltration or lyophilization. Denatured material was removed by brief centrifugation and samples were loaded on C_{18} μBondapak reverse-phase high-performance liquid columns (0.39 x 30 cm). Proteins were eluted in 15-45% or 10-60% gradients of acetonitrile containing 0.01 M trifluoroacetic acid (TFA) and the column flow rate was 0.5-1.0 ml/min. The eluant was monitored for relative absorbance at 215 nm and the fractions were tested for the presence of mitogenic activity on AKR-2B cells.

Cell Proliferation Assay

AKR-2B cells were seeded at 3.15 x 10^5 cells/60 mm culture dish, with each dish containing 5 ml of DMEM plus 0.5% CS. The cells were then incubated in a humidified 5% CO_2 atmosphere at 37°C in the presence and absence of partially purified ULF mitogen [ULFM; Sephadex G-50(f) fraction] added at a concentration of 150 ng/dish. Cells were refed every other day with DMEM or DMEM plus ULFM, and cell viability and morphology were monitored under the microscope. For determination of cell number, cells were detached from plates with ATV solution and suspensions were counted using a hemocytometer (American Optical Corp., Buffalo, NY).

Characterization of the ULF Mitogen

Growth factor sensitivity to proteolytic digestion was tested by incubation of the partially purified ULFM with bovine pancreatic trypsin (500 μg/ml) at 37°C for 4 h. Trypsin was then inactivated by subsequent addition of soybean trypsin inhibitor (500

μg/ml) to the reaction mixture. The control consisted of an equivalent amount of the factor, trypsin, and trypsin inhibitor mixed prior to the start of the incubation. Digestion with proteinase K (100 μg/ml; Boehringer-Mannheim, Indianapolis, IN) was carried out at 37°C for 4 h and then terminated by heating at 100°C for 5 min. Activity remaining was compared to that observed with the factor and proteinase K heated to 100°C for 5 min prior to the 4 h incubation at 37°C. Treatment of the mitogen with 2% β-mercaptoethanol was carried out for 24 h at 4°C and the sample was then dialyzed against three changes of PBS for 24 h at 4°C. The effect of urea on the mitogen was evaluated at a final concentration of 6 M urea at 4°C for 24 h, after which the sample was dialyzed against three changes of PBS overnight at 4°C. The control sample was incubated for 24 h at 4°C and dialyzed in parallel with the urea and β-mercaptoethanol-treated samples. All samples were then tested for mitogenic activity using density-arrested AKR-2B cells.

Statistical Analysis

Values are shown as the means ± SEM. Results were compared for statistical significance using Student's paired t-test (Steel and Torrie, 1980). Analysis of variance using the General Linear Models Procedure of the Statistical Analysis System (Barr et al., 1979) was performed to detect the effect of day, treatment, and their interaction on AKR-2B cell proliferation.

Results

Unfractionated uterine luminal fluid (ULF) from Day 12 pregnant gilts was concentrated by ultrafiltration and tested for mitogenic activity by its ability to stimulate [^{3}H]thymidine incorporation into DNA of several well-characterized indicator cell lines. This mitogen assay was previously shown to correlate with the cell proliferation activity of known growth factors in several other systems (Carpenter and Cohen, 1976; Brown and Blakeley, 1984). The crude ULF sample stimulated mitogenesis of AKR-2B fibroblasts and MDCK epithelial cells in a dose-dependent manner, but failed to stimulate DNA synthesis of A431 epidermoid carcinoma cells (Table 2-1). Since AKR-2B cells exhibited the maximal mitogenic response to crude ULF ($P<0.01$), these cells were used to further study the resident mitogenic components.

To characterize the factor(s) responsible for ULF activity, ULFs were subjected to chromatography on a Sephadex G-200 gel-filtration column. Aliquots of each eluted fraction were then tested for mitogenic activity by monitoring incorporation of [^{3}H]thymidine into DNA of quiescent AKR-2B fibroblasts. Figure 2-1 (panel A) shows that the mitogenic activity eluted as a single peak with a M_r less than the 17,000 M_r standard. Rechromatography of the pooled active fractions on a Sephadex G-50 column using additional protein standards (bovine insulin of M_r 5,700) indicated an approximate native M_r of 4,800 (Figure 2-1, panel B).

Table 2-1. Mitogenic activity of pooled uterine luminal fluids from Day 12 pregnant gilts using different indicator cell lines.[a]

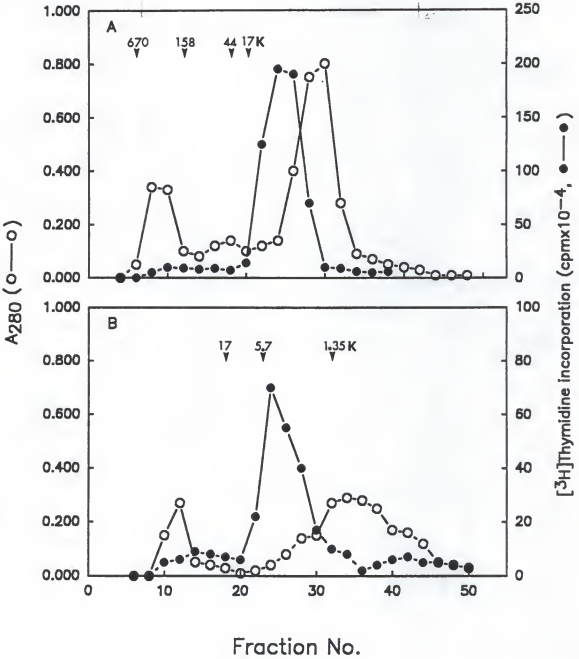
Cell line[b]	Amount (μg protein)[c]	Relative [^{3}H]thymidine incorporation[d]
AKR-2B	0	1.0
	50	7.9**
	200	10.7**
MDCK	0	1.0
	50	2.2*
	200	3.7**
A431	0	1.0
	50	1.0
	100	1.0

[a] ULFs from six Day 12 pregnant gilts were pooled.
[b] AKR-2B (=mouse embryo-derived fibroblast cells), MDCK (=Madin-Darby canine kidney epithelial cells) and A431 (= human A431 epidermoid carcinoma cells).
[c] Protein concentrations were determined using the Bradford protein assay.
[d] Values are the ratio of increase in [^{3}H]thymidine incorporated into the DNA of quiescent cells in the presence of added sample (n=3) over that incorporated by cells which received an equivalent volume of PBS.
* $P<0.05$ and ** $P<0.01$

Figure 2-1. Gel-filtration chromatography of uterine luminal fluid (ULF) from Day 12 pregnant gilts.

(A) ULF (22.5 mg) was chromatographed on a Sephadex G-200 column (2.5 x 45 cm) preequilibrated with PBS (pH 7.4). The flow rate was 20 ml/h and 3 ml fractions were collected. Aliquots (200 μl) of every other fraction were tested in triplicate for mitogenic activity on AKR-2B cells, as described in Materials and Methods.

(B) Fractions (# 20-30) from the Sephadex G-200 run were concentrated, reapplied to a Sephadex G-50 column (2.5 x 50 cm), and processed as in (A). Arrows indicate the elution position of column M_r standards. The protein elution profile at absorbance 280 nm is shown by (o-o) and the mitogenic activity profile on mouse embryo-derived fibroblast (AKR-2B) cells by (•--•).


A
670
158
44
17K
B
17
5.7
1.35K
A280 (o—o)
[3H]Thymidine Incorporation (cpmx10−4, ●—●)
Fraction No.

Other ULF samples from pregnant and cyclic gilts were also examined for the presence of low M_r mitogenic activity to correlate such expression with the physiological state of the animal (Figure 2-2). ULFs obtained from Day 10 pregnant (panel A) and from Day 12 cyclic (panel B) gilts were fractionated using a Sephadex G-200 column, and the presence of a similar mitogenic activity in both ULF samples was detected. Although it was not possible to precisely quantify the relative amounts present in pregnant and cyclic gilt uterine secretions, these results suggest that the mitogenic factor(s) is not unique to the ULF of pregnant gilts. The presence of similar mitogenic activity was not detected in cytosolic extracts prepared from uterine tissue (panel C) or serum (panel D) from Day 12 pregnant gilts. No significant activity comparable to that in ULF was detected in fractionated uterine cytosol, whereas mitogenic activity in gilt serum was confined to high M_r proteins.

Since the M_r (4,800) of the mitogenic factor(s) is similar to that of EGF or TGF-α (M_r of 6,000), the possibility that the mitogen(s) identified in ULF corresponds to porcine EGF or an EGF analogue was examined using rabbit immunoglobulin G (IgG) specific for mEGF. Figure 2-3 shows that anti-mEGF IgG (50 μg) inhibited the stimulatory effect of crude unfractionated ULF from Day 12 pregnant gilts on AKR-2B fibroblasts. The effect was not total (60% inhibition at the antibody concentration used), but appeared to be antibody-specific, since an equivalent amount of control antibody (IgG raised against an unrelated protein) did not reduce the [^{3}H]thymidine incorporation by cells receiving added ULF. In addition, increasing the amount of anti-EGF antibody used to 100 μg did not further increase

Figure 2-2. Mitogenic activities of pregnant and cyclic gilt ULFs, uterine cytosolic extract and maternal serum on AKR-2B cells.

ULFs from Day 10 pregnant (38 mg, panel A) and Day 12 cyclic gilts (25.6 mg, panel B), cytosolic extract from the uteri of Day 12 pregnant gilts (40 mg, panel C), and serum from Day 12 pregnant gilts (36 mg, panel D) were separately chromatographed on a Sephadex G-200 column (2.5 x 45 cm) using PBS as eluent at a flow rate of 20 ml/h. Aliquots (200 μl) of every other fraction were assayed in triplicate for mitogenic activity (•--•). Arrows indicate the elution position of M_r markers and the protein elution profile at A_{280} is shown by (o-o).

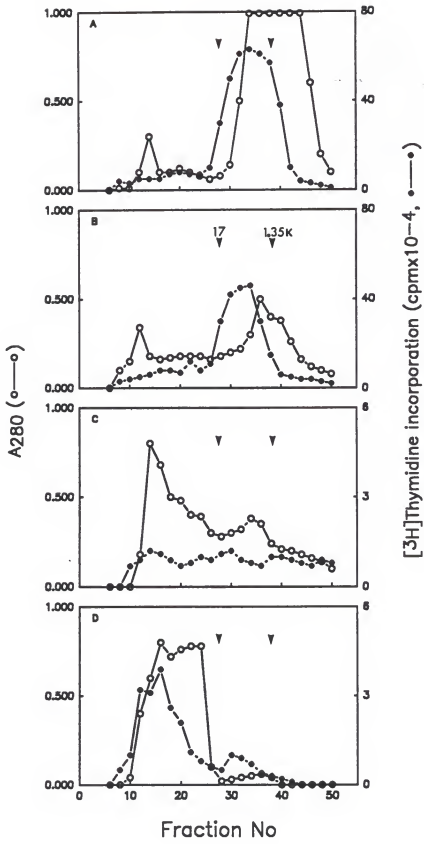
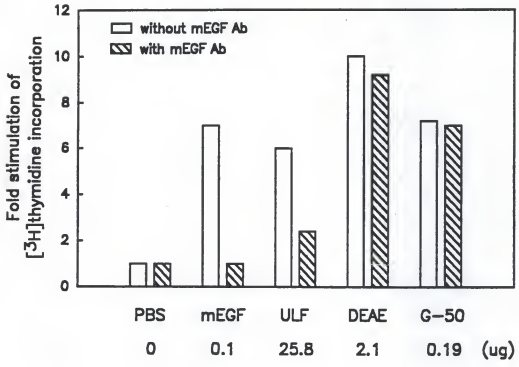

A
B
C
D
17
1.35K
A280 (o—o)
[3H]Thymidine incorporation (cpmx10−4, •—•)
Fraction No
1.000
0.500
0.000
80
40
0
6
3
0
10
20
30
40
50

Figure 2-3. Evidence that the uterine luminal fluid mitogen (ULFM) is distinct from EGF.

The mitogenic activities of unfractionated ULF and of the partially purified mitogen [DEAE ion-exchange and Sephadex G-50(f) fractions (ULFM)] were assayed in the absence (white bars) and presence (diagonally striped bars) of anti-mouse EGF (IgG, 50 μg). The effect of the antibody on mEGF (100 ng) is presented as a positive control. Results are expressed as relative-fold stimulation of [^{3}H]thymidine incorporation into DNA of AKR-2B cells over PBS.


12
10
8
6
4
2
0
without mEGF Ab
with mEGF Ab
Fold stimulation of
[3H]thymidine incorporation
PBS
mEGF
ULF
DEAE
G-50
0
0.1
25.8
2.1
0.19
(ug)

its inhibitory effect (data not shown). These results suggest that crude ULF contains EGF or an EGF analogue that is responsible for the reduction in its mitogenic activity with anti-EGF IgG and that the 4,800 M_r factor(s) (Figure 2-1) is antigenically related to mEGF.

To examine the latter possibility, partial purification of the mitogenic activity was undertaken. For purification of the ULF factor, unfractionated ULFs collected from Day 10 and/or Day 12 pregnant gilts were concentrated by ultrafiltration, placed in boiling water for 7 min, and centrifuged to remove denatured/precipitated proteins. Heat treatment did not to reduce the mitogenic activity of the ULF factor (Table 2-4), but removed approximately 60% of the total protein in crude ULF (Table 2-2). The supernatant fraction was then applied to a Sephadex G-50(c) column preequilibrated with PBS (pH 7.4). Figure 2-4 shows that highest M_r proteins (approximately 80% of total proteins present in heat-treated ULF) were removed at this step. The mitogenically active fractions were further fractionated through a DEAE anion-exchange chromatography (Figure 2-5). In general, 70-80% of the resident mitogenic activity in Sephadex G-50(c) peak fractions was quantitatively bound by DEAE in low ionic strength buffer and subsequently eluted with an increasing salt concentration gradient. The first active fractions eluted between 0.3-0.4 M NaCl, corresponding to the major mitogenic activity, possessing 50% greater specific activity than the other fractions. This peak of acidic proteins was dialyzed against PBS and chromatographed on a Sephadex G-50(f) gel-filtration column, and aliquots of the fractions were tested for their ability to stimulate DNA synthesis

Figure 2-4. Gel-filtration chromatography of pooled ULFs from Day 10 and 12 pregnant gilts.

Heat-treated ULF (287 mg) was concentrated by ultrafiltration and applied to a Sephadex G-50(c) column (5 x 100 cm) preequilibrated with PBS (pH 7.4). The flow rate was 45 ml/h and 14 ml fractions were collected. Aliquots (100 μl) of fractions were tested (n=3) for mitogenic activity (Materials and Methods). Active peak fractions (# 74-104) were pooled and used for further purification. Protein elution at absorbance 280 nm is shown by (o-o) and mitogenic activity by (•--•).

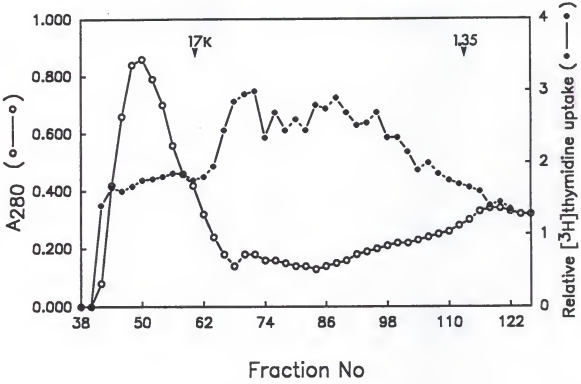

1.000
0.800
0.600
0.400
0.200
0.000
A280 (o——o)
17K
135
4
3
2
1
0
Relative [3H]thymidine uptake (•——•)
38
50
62
74
86
98
110
122
Fraction No

Figure 2-5. Anion-exchange chromatography of pooled active peak fractions from the Sephadex G-50(c) column.

Active peak fractions from the previous Sephadex G-50(c) run were pooled, concentrated, dialyzed against 0.01 M Tris (pH 8.3), and loaded (100 mg) on a DE-52 cellulose anion-exchange column (14 x 1.7 cm). The flow rate was 30 ml/h and 6 ml fractions were collected. A continuous gradient of 0-3 M NaCl in 0.01 M Tris (pH 8.3) was then used to elute bound proteins. Eluted proteins were separately pooled (fractions 107-128, 129-137, 138-156), concentrated, and tested for the ability to stimulate DNA synthesis of AKR-2B cells. Values with the hatched bars are the fold increase in specific activity with the same amount of proteins (0.8 μg) over control (0.01 M Tris only). The active peak fractions (# 107-128) were utilized for further fractionation. Fractions were monitored for absorbance at 280 nm (o-o). Relative mitogenic activity of each peak is shown by values above the hatched bar and NaCl gradient is indicated by (---).

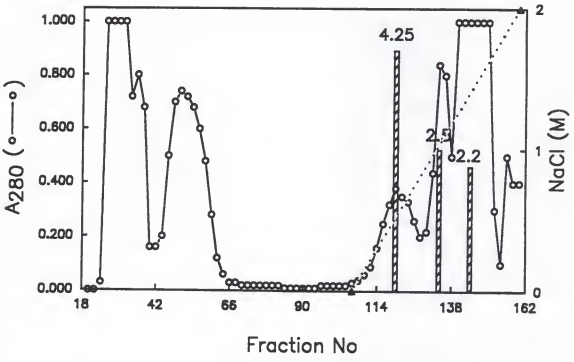
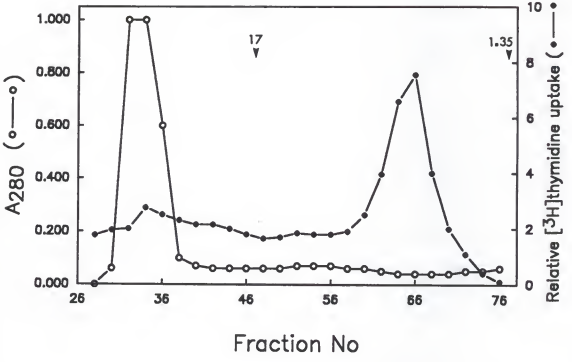

1.000
0.800
0.600
0.400
0.200
0.000
A280 (o—o)
18
42
66
90
114
138
162
Fraction No
2
1
0
NaCl (M)
4.25
2.5
2.2

Figure 2-6. Gel-filtration chromatography of active peak fractions from DEAE ion-exchange chromatography.

The mitogenically active peak fraction from the DEAE run was dialyzed against PBS (pH 7.4) and applied (26 mg) to a Sephadex G-50(f) column (2.5 x 50 cm) preequilibrated with PBS (pH 7.4). The flow rate was 40 ml/h and 3 ml fractions were collected. The protein elution profile at A_{280} is represented by (o-o) and the analysis of mitogenic activity of 50 μl aliquots on AKR-2B cells is shown by (•--•). Active peak fractions (# 60-69 were pooled for a final purification step.


A280 (o—o)
1.000
0.800
0.600
0.400
0.200
0.000
17
1.35
10
8
6
4
2
0
Relative [3H]thymidine uptake (•—•)
26
36
46
56
66
76
Fraction No

(Figure 2-6). Almost all of the activity was found to elute in the region between myoglobin (M_r of 17,000) and vitamin B-12 (M_r of 1,350), demonstrating the size-correspondence of the mitogenic activity in ULF and the DEAE fraction (Figures 2-1 and 2-5). These active fractions were pooled and analyzed by reverse-phase high performance liquid chromatography (RP-HPLC). Figure 2-7 demonstrates the presence of two factors which differ in hydrophobicity. A hydrophilic peak eluted between 10 and 15% of acetonitrile (CH_3CN) and a relatively hydrophobic factor eluted at ~30% of CH_3CN. The specific activity of the hydrophilic factor was about 40% greater than that of the hydrophobic factor. The relative amount of both proteins was estimated at 1% (1 μg) of total proteins loaded (100 μg) on the reverse-phase column. A preliminary study using mEGF as a standard revealed that mEGF eluted between 25 and 35% of CH_3CN (data not shown).

Table 2-2 summarizes the purification scheme used and the fold-purification obtained. This protocol using sequential chromatographies achieved an approximately 1,100-fold partial purification of the ULF mitogenic factor(s).

The mitogenic activity of partially purified ULFM [Sephadex G-50(f) fraction] was then examined for the effect of added anti-EGF IgG. Figure 2-3 demonstrates that, whereas unfractionated ULF activity was significantly reduced by anti-EGF IgG, the activities of the DEAE and the Sephadex G-50(f) fractions were not different in the presence or absence of the antibody. This result indicates that the partially purified 4,800 M_r ULFM is antigenically distinct from mEGF. The inability of

Figure 2-7. Reverse-phase high performance liquid chromatography (RP-HPLC) analysis of the mitogenic fractions from the Sephadex G-50(f) column run.

Approximately 100 μg of concentrated active Sephadex G-50(f) fractions was loaded onto a C_{18} μBondapak RP-HPLC column (0.39 x 30 cm) and subsequently eluted in a 15-45% gradient of acetonitrile in 0.01 M TFA (---). Flow rate was 0.5 ml/min and protein elution was monitored at A_{215}. Fractions of 1 ml were collected, lyophilized, and reconstituted in 200 μl of H_2O. Mitogenic activity of each fraction for AKR-2B cells was determined in duplicate using 10 μl aliquots, as described in Materials and Methods. The value above each bar represents fold-stimulation of [^{3}H]thymidine incorporation by AKR-2B cells over control (H_2O only).

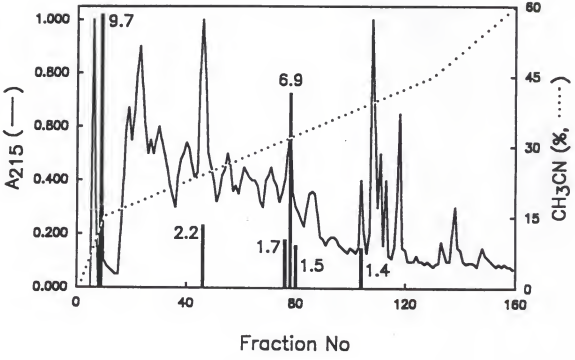

1.000
0.800
0.600
0.400
0.200
0.000
A215 (—)
9.7
6.9
2.2
1.7
1.5
1.4
0
40
80
120
160
Fraction No
60
45
30
15
0
CH3CN (%, ·····)

Table 2-2. Partial purification of uterine luminal fluid mitogen (ULFM).[a]

	Total protein (mg)	Total activity (units)[b]	Specific activity (U/mg)	Recovery (%)[c]	purification[d] (fold)
Crude	3362.0	109620	32.6	100	1.0
Heating	1436.0	67000	46.7	61.1	1.4
G-50 (coarse)	275.4	26493	96.2	24.2	3.0
DEAE	29.8	4350	146.0	4.0	4.5
G-50 (fine)	.487	1140	2340.1	1.0	71.8
RP-HPLC[e]	.010	361	36100.0	0.3	1107.4

[a] ULFs were collected from gilts (n=47) in Days 6-18 pregnancy.

[b] Activity was determined by monitoring [^{3}H]thymidine incorporation into DNA of density-arrested, quiescent AKR-2B cells, as described in Materials and Methods. One unit of activity is defined as an amount of factor which half-maximally stimulates DNA synthesis in the mitogen assay.

[c] Recovery is defined as the percentage of total activity at each step over that of crude ULF.

[d] Fold-purification is defined as the ratio of specific activity at each step to that of crude ULF.

[e] Protein amount was calculated by comparing an elution peak area of the factor with that of known amount of EGF after RP-HPLC.

increasing concentrations of the DEAE fraction to displace [^{125}I]mEGF binding to A431 cells further supports these results (Simmen et al., 1988c).

DNA synthesis in A431 cells is inhibited ($P<0.01$) by ng/ml concentrations of EGF (Barnes, 1982; Kawamoto et al., 1983; Table 2-3). In contrast, partially purified ULFM [Sephadex G-50(f) fraction] at different concentrations stimulated DNA synthesis ($P<0.01$) in these cells in a dose-dependent manner (Table 2-3). On the other hand, ULFM was not mitogenic for MDCK cells at 250 ng/ml, but it stimulated DNA synthesis at 500 ng/ml ($P<0.05$) (Table 2-3).

Table 2-3. [^{3}H]Thymidine incorporation by human A431 epidermoid carcinoma cells and Madin-Darby canine kidney cells (MDCK) when stimulated by the indicated amounts of ULFM.

Cells	Sample	Conc (ng/ml)	[^{3}H]thymidine incorporation (cpm x 10^{-3})[a]
A431	ULFM	0	275 ± 40
		60	653 ± 34**
		120	737 ± 6**
		250	947 ± 2**
	mEGF	0	362 ± 19
		50	150 ± 1**
MDCK	ULFM	0	3.8 ± 0.3
		250	3.8 ± 0.05
		500	4.5 ± 0.3*

[a] Values are the means ± SEM (n=3).
* $P<0.05$ and ** $P<0.01$

Table 2-4 presents a summary of the effects of proteolytic enzymes, denaturing conditions, reducing agents, and heat on the mitogenic activity of the partially purified ULFM. This mitogen appears to be a polypeptide since its activity was abolished by incubation with trypsin and proteinase K. The mitogen is extremely stable to heat treatment, maintaining 100% of its activity after 7 min at 100°C. Finally, the activity of the factor was unaffected by the dissociating agent urea possibly due to a renaturation of protein during dialysis; however, it was totally abolished upon reduction by ß-mercaptoethanol. In addition, the use of anion-exchange HPLC revealed that ULFM is an acidic (pI of 6.4) polypeptide (Y Ko and FA Simmen, unpublished observation).

Table 2-4. Characterization of porcine ULFM.

Treatments[a]	Activity remaining (%)
Trypsin, 37°C, 4 h	11%
Trypsin + Trypsin inhibitor, 37°C, 4 h	100%
Proteinase K, 37°C, 4 h	0%
6 M urea, 4°C, 24 h	100%
2% (v/v) ß-mercaptoethanol, 4°C, 24 h	0%
100°C, 7 min	100%

[a] The ULF Sephadex G-50(f) fraction (ULFM; 0.67 μg) was treated according to the above protocols as described under Materials and Methods. Results are expressed as the activity remaining relative to the same concentration of appropriate controls.

Figure 2-8 shows the effects of partially purified ULFM on the growth of AKR-2B fibroblastic cells plated at a low density in DMEM containing 0.5% CS. Cells grown in the presence of the factor (150 ng total protein) grew to a density 50% greater ($P<0.01$) than those in the presence of medium plus 0.5% CS alone. The effect of day was apparent ($P<0.01$) up to 3 days of treatment and the interaction of day X treatment was detected ($P<0.01$).

The mitogenic activity of ULFM on primary cultures of uterine cells was next investigated as an initial step towards determining its physiological role in vivo. Partially purified ULFM was added at varying concentrations to stromal cells isolated from uteri of Day 12 pregnant sows. Stimulation of DNA synthesis was monitored by the relative increase in [^{3}H]thymidine incorporation over PBS. Figure 2-9 indicates that the incorporation of [^{3}H]thymidine in quiescent confluent cultures of stromal cells was increased approximately 3-fold upon addition of ULFM. The response was dose-dependent and was not inhibited by anti-mEGF IgG. These cells responded in a mitogenic fashion to EGF and human IGF-I (ng/ml range) under our assay conditions (Figure 2-9).

Discussion

This study describes the partial purification and initial characterization of a fibroblast mitogen, termed uterine luminal fluid mitogen (ULFM), in ULFs of early pregnant and cyclic gilts. This factor, like EGF, stimulates mitogenesis in fibroblastic

Figure 2-8. Effect of ULFM on the proliferation of mouse embryo-derived fibroblast (AKR-2B) cells.

Cells (3.15×10^5) were seeded in 60 mm culture dish in 5 ml of DMEM containing 0.5% calf serum and incubated at 37°C in an atmosphere of 5% CO_2 in the absence (o-o) and presence (•••) of ULFM (150 ng per dish). Cells were refed every other day with either DMEM or DMEM plus ULFM. At indicated times, duplicate dishes were removed and the cell numbers were determined. Data are shown as the mean cell counts ± SEM.

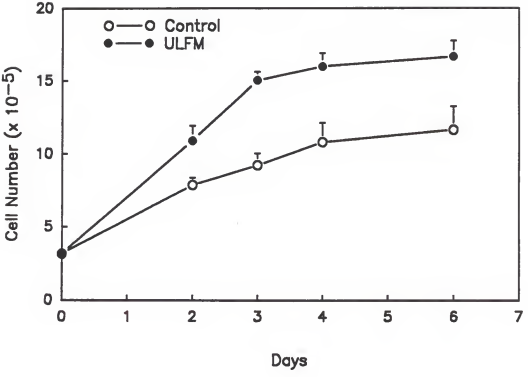
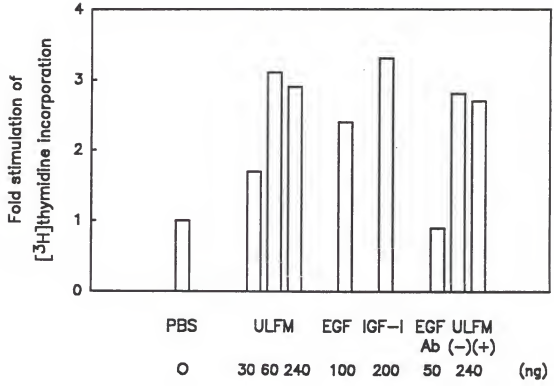

Control
ULFM
Cell Number (x 10⁻⁵)
20
15
10
5
0
0
1
2
3
4
5
6
7
Days

Figure 2-9. Effect of ULFM on [^{3}H]thymidine incorporation into DNA of porcine uterine stromal cells.

Uterine stromal cells were utilized in mitogen assays, as described in Materials and Methods. Open bars represent the ratio of [^{3}H]thymidine incorporated by cells in the presence of indicated amounts of ULFM, mouse epidermal growth factor, or human insulin-like growth factor-I over that incorporated by cells receiving an equivalent volume of PBS. Anti-mouse EGF IgG (50 μg/ml) was added to cells in the absence (-) or presence (+) of ULFM.


Fold stimulation of
[3H]thymidine incorporation
4
3
2
1
0
PBS
ULFM
EGF
IGF-I
EGF Ab
ULFM (-)(+)
0
30 60 240
100
200
50
240
(ng)

(AKR-2B and uterine stromal) cells, is a small polypeptide of approximately 4,800 M_r and is extremely heat stable. However, ULFM is distinct from EGF on the basis of immunological and biochemical analyses of its activity. First, ULFM activity is not neutralized by the addition of antibody specific for mouse EGF, indicating immunological unrelatedness of ULFM with EGF. Second, ULFM does not compete for the binding of [^{125}I]mEGF to A431 cell EGF receptors, suggesting distinct receptors for ULFM in target cells. Third, EGF does not stimulate DNA synthesis in A431 cells that respond to ULFM in a dose-dependent manner.

ULFM is not likely to be the only growth-promoting activity in porcine uterine secretions. The results regarding the inhibitory effect of anti-EGF IgG on unfractionated ULF mitogenic activity suggest that EGF or EGF-related molecules are also components of ULF. Similarly, the presence of an EGF-like factor in rat uterine luminal fluids has been reported (Imai, 1982). Other growth factors, such as the IGFs, have been identified in uterine secretions of pigs (Simmen et al., 1989b; Letcher et al., 1989a; Simmen and Simmen, 1990), sheep (Ko et al., 1991), and cows (Geisert et al., 1991). FGFs were also detected in pig ULFs (Brigstock et al., 1989). In addition, anion-exchange and gel-filtration chromatography of ULF demonstrated several other fractions that exhibit mitogenic activity towards AKR-2B cells. Chromatography of unfractionated ULF on Sephadex G-200 gel filtration columns also revealed the presence of a broad AKR-2B mitogen peak, indicating mitogenic activities with similar M_rs to ULFM. However, these proteins appear to have different hydrophobicity from ULFM, as demonstrated from the protein elution

profile obtained by HPLC. Finally, partially purified ULFM cannot account for the mitogenic activity of crude ULF on MDCK (epithelial) cells.

In this study, the presence of mitogenic activity in ULF was detected on the basis of its ability to stimulate [^{3}H]thymidine incorporation by cells in culture. This assay, although relatively nonspecific with respect to growth factor type, has been shown to correlate with cell proliferation activity of known peptide growth factors in other systems. Indeed, this factor can promote the proliferation of fibroblastic (AKR-2B) cells, confirming its identity as a true mitogen. ULFM exhibited cell type specificity in its activity, being stimulatory to AKR-2B and A431 cells as well as primary cultures of uterine stromal cells but not to MDCK cells. On the basis of its chromatographic properties, ULFM is distinct from TGF-ß (M_r of 25,000), PDGF (28,000-31,000), and FGFs (16,000-18,000). ULFM also appears to be distinct from TGF-α (5,700-6,000), since it was noncompetitive in A431 EGF receptor binding (Simmen et al., 1988c). Finally, ULFM does not represent the free form of IGF-I since the latter is nonmitogenic in the AKR-2B assay system (Simmen et al., 1988a).

Several approaches were used in attempts to further purify ULFM. These include RP-HPLC, Mono Q anion-exchange HPLC (Pharmacia LKB Biotechnology, Piscataway, NJ) and polypropyl aspartamide hydrophobic interaction HPLC (Nest group, Southboro, MA), all of which were unsuccessful. The final purification achieved was 1,100-fold with respect to specific activity, although the total amount of protein removed was substantially greater. Similar to conceptus secretory proteins of domestic animals (Godkin et al., 1982a, 1982b; Helmer et al., 1987), ULFM may

exist in its native form, not as a single polypeptide, but as complexed with other proteins, all of which contribute to a stable conformation essential for activity. Although ULFM was not purified to homogeneity, results of the present study suggest that ULFM is a novel growth factor distinct from other known growth factors known to date.

The apparent specific accumulation of ULFM in ULF raises the question of its tissue origins and/or mechanism of transport. Several studies utilizing techniques of immunohistochemistry, in situ hybridization and Northern blot analysis have demonstrated local production of growth factors in the uterus. These factors include the IGFs (Simmen and Simmen, 1990), EGF (Popliker et al., 1987), CSF-1 (Arceci et al., 1989), and FGFs (Brigstock et al., 1989). By analogy, ULFM may also be of uterine origin. On the other hand, ULFM is not likely to be of conceptus origin, since the presence of ULFM in ULF of cyclic gilts was detected. Alternatively, the presence of ULFM in ULF may result from transcellular (receptor-mediated) or paracellular transfer of plasma-derived growth factor. In this regard, ULF is known to contain other proteins of both plasma and uterine tissue origins (Kuivanen and DeSombre, 1985).

The physiological functions of growth-promoting activities in porcine uterine secretions are also unknown. IGFs are postulated to act as local mediators of porcine conceptus and uterine development via autocrine and/or paracrine routes (Simmen and Simmen, 1990, 1991; Hofig et al., 1991a, 1991b). Similarly, the regulation of embryonic and uterine growth in vivo and in vitro by EGF (Tomooka

et al., 1986; Paria and Dey, 1990), TGF-α (Dore et al., 1991), TGF-β (Kauma et al., 1990), and FGFs (Hondermarck et al., 1990) has been suggested. Therefore, it is possible that ULFM may play a similar role, in concert with other growth factors and hormones. In pregnant gilts, the expression of ULFM in ULF decreases with increases in levels of ULF IGF-I and systemic progesterone (Simmen et al., 1989a), suggesting a possible role for ULFM in the regulation of endometrial IGF-I expression and/or a negative regulation by progesterone of ULFM expression. Although ULFM was shown to stimulate mitogenesis in uterine stromal cells cultured in vitro, its effects on uterine epithelial cells are as yet unknown. Nevertheless, ULFM activity towards both fibroblastic (AKR-2B and uterine stromal) and epithelial-like (A431) cells suggests possible regulatory actions of this factor on both epithelial and fibroblastic cells of the uterus.

CHAPTER 3
INSULIN-LIKE GROWTH FACTORS IN SHEEP UTERINE FLUIDS: EXPRESSION AND RELATIONSHIP TO OVINE TROPHOBLAST PROTEIN-1 PRODUCTION

Introduction

The potential importance of uterine luminal fluids (ULFs) in conceptus-maternal communication and in the regulation of peri-implantation conceptus development has been emphasized (Roberts & Bazer, 1988; Brigstock et al., 1989; Simmen & Simmen, 1990, 1991). Molecules of uterine or conceptus origin in ULF include polypeptide growth factors (Simmen et al., 1988c; Brigstock et al., 1989; Simmen & Simmen, 1991), enzymes (Mullins et al., 1980; Farmer et al., 1990), progesterone-induced transport proteins (Simmen et al., 1988b; Harney et al., 1990), and interferon-related trophoblast secretory proteins (Godkin et al., 1982a, 1982b; Helmer et al., 1987; Gnatek et al., 1989). The synthesis and secretion of these molecules is under hormonal and/or developmental regulation (Roberts and Bazer, 1988; Simmen and Simmen, 1990).

Maternal recognition of pregnancy is believed to be signalled by conceptus estrogens in the pig (Bazer and Thatcher, 1977), by ovine trophoblast protein-1 (oTP-1) in the sheep (Godkin et al., 1984a), by caprine trophoblast protein-1 (cTP-1) in the goat (Gnatek et al., 1989), and by bovine trophoblast protein-1 (bTP-1) in the

cow (Helmer et al., 1987). Based upon the apparent complexity of physiological and biochemical interactions in the uterine environment around the time of maternal recognition of pregnancy, it is likely that these factors exert their effects in concert with other developmentally regulated ULF proteins. In this regard, amounts of insulin-like growth factor (IGF)-I in ULF of pregnant and cycling pigs are maximal at Day 12, coincident with maternal recognition of pregnancy (Geisert et al., 1982b; Letcher et al., 1989a; Simmen et al., 1989a). Similarly, IGF-I mRNAs in pig uterine endometrium are most abundant at Day 12 of pregnancy (Letcher et al., 1989a; Simmen et al., 1992).

Ovine TP-1 secreted by the sheep conceptus has antiluteolytic properties (Godkin et al., 1984b). The synthesis and secretion of oTP-1 mRNA and protein by the conceptus are temporally associated with maternal recognition of pregnancy (Farin et al., 1989; Guillomot et al., 1990). The secretion of specific endometrial proteins are amplified during this period, suggesting a direct or indirect relationship to secretion of oTP-1 by the conceptus (Godkin et al., 1984a; Vallet al., 1987; Sharif et al., 1989; Ashworth & Bazer, 1989). The specific physiological and temporal relationships of oTP-1 and peptide growth factors (known regulators of gene expression and protein secretion) in the uterine lumen have not been previously examined. Therefore, both pigs and sheep have common characteristics: conceptuses undergo rapid morphological changes during peri-implantation period and both have a noninvasive type of placentation. Uterine IGF-I has been shown to be an important regulatory factor for porcine conceptus development during the period of

maternal recognition of pregnancy (Simmen and Simmen, 1990). In this study, uterine secretions from cycling (C) and pregnant (Px) ewes were analyzed for presence of IGFs and for presence of mitogenic factors distinct from the IGFs. Finally, in vitro effects of recombinant IGFs on oTP-1 secretion from conceptuses were investigated.

Materials and Methods

Animals

Adult crossbred ewes, primarily of Rambouillet breeding, were checked daily for estrous behavior using vasectomized rams. Ewes were bred with an intact ram at the first sign of estrus (Day 0) and thereafter at approximately 12 h intervals, until cessation of estrus.

Materials

All tissue culture media and reagents, goat anti-mouse IgG antiserum, and normal mouse serum were purchased from Sigma Chemical Co. (St. Louis, MO). Calf serum was obtained from GIBCO (Grand Island, NY), and tissue culture petri dishes and multiwell plates were from Corning Glass Works (Corning, NY) and Becton-Dickson (Lincoln Park, NJ), respectively. Bradford protein assay reagents and gel-filtration M_r standards were from Bio-Rad (Rockville Centre, NY). Recombinant human insulin-like growth factor (rhIGF)-I and -II were purchased from Amgen Biologicals (Thousand Oaks, CA) and mouse monoclonal IgG specific to rat IGF-II was from Amano (Troy, VA). Rabbit anti-human platelet-derived

growth factor (PDGF) antiserum was from Collaborative Research (Bedford, MA). Iodogen was purchased from Pierce (Rockford, IL), and free ^{125}I and [^{3}H]thymidine (21 mCi/mg) were from Amersham (Arlington Heights, IL). Sep-Pak^{R} plus C_{18} cartridges were from Waters (Milford, MA).

Collection of Uterine Luminal Fluids

Anesthesia was induced and maintained with methoxyflurane administered via a closed circuit gas anesthetic unit. The reproductive tract was exposed via midventral laparotomy under aseptic conditions. ULFs from ewes [Days 10, 12, 14 and 16 of the estrous cycle (C) and pregnancy (Px); n=4/day/status except for Day 10 C/Px and 14 Px (n=3) and Day 12 Px (n=2); total of 27] were collected by flushing each horn with 20 ml of sterile 0.9% saline solution. Pregnancy was confirmed by the presence of an apparently normal conceptus in the flushing. ULFs were transferred to sterile tubes, centrifuged (1,000 x g for 15 min) to remove insoluble materials, and stored at -20°C until analyzed. Protein content in ULF was measured by the Bradford protein assay (Bradford, 1976) using bovine serum albumin as the standard.

Radioimmunoassays (RIAs)

Both recombinant human IGF-I (^{59}Thr) and IGF-II were iodinated to specific activities of 150-350 μCi/μg protein using Iodogen. Iodinated IGFs were purified on a Sephadex G-50 column and stored at -20°C. Sep-Pak^{R} plus C_{18} cartridges were used to facilitate removal of IGF-binding proteins (IGFBPs) from ULF or conceptus culture media prior to IGF-I and -II RIAs as previously reported (Lee et al., 1991).

In brief, 1.2 ml ULF or conceptus culture media was acidified with 1.8 ml 1% aqueous trifluoroacetic acid (TFA) for 10 min at 24°C and loaded on the cartridge which was pre-equilibrated with sequential washes of 100% acetonitrile (CH_3CN), distilled water, and 0.1% TFA in H_2O. Cartridges were washed with 3 ml 0.1% TFA, and the retentate was eluted in 100% CH_3CN containing 0.1% TFA, air-dried in a 37°C water bath and solubilized in 0.48 ml RIA buffer (30 mM sodium phosphate, 0.02 % protamine sulfate, 10 mM EDTA, 0.05% Tween-20, 0.02% sodium azide; pH 7.5). An aliquot (0.1 ml) was incubated with IGF-I rabbit antiserum (UBK 487, National Hormone and Pituitary Program, final dilution of 1:20,000) and 15,000-20,000 cpm [^{125}I]IGF-I in RIA buffer (total volume of 0.5 ml) for 16 h at 4°C. Sheep antiserum (0.1 ml) to rabbit gamma globulin and 0.1 ml normal rabbit serum (1:100 dilution) were added, and the mixture incubated for 1 h at 4°C. After addition of 1 ml RIA buffer, tubes were centrifuged at 3,000 rpm for 30 min at 4°C. The supernatant was aspirated and radioactivity in the pellet counted. IGF-II RIA was carried out using 0.1 ml of the solubilized CH_3CN eluate from the cartridge, 0.2 ng mouse monoclonal IgG against rat IGF-II, and 15,000-20,000 cpm [^{125}I]IGF-II in RIA buffer under the same incubation conditions described above. The antigen-antibody complexes were precipitated using 0.1 ml goat antiserum (1:10 dilution) to mouse IgG and 0.1 ml normal mouse serum (1:100 dilution). Recombinant human IGF-I and IGF-II were used as reference standards and IGF content was determined from logit-log plots. All ULF samples were assayed at the same time to avoid inter-assay variation. The minimal detectable dose and half-maximal displacement occurred at

5 pg (5-8% displacement) and 55-65 pg unlabeled IGF-I in the IGF-I RIA, and at 20 pg (7-13% displacement) and 90-110 pg unlabeled IGF-II in the IGF-II RIA, respectively. Intra-assay coefficient of variation was 4.9% in the IGF-I RIA and 5.5% in the IGF-II RIA.

An RIA previously developed by Vallet et al. (1988a) was used to quantitate oTP-1 in conceptus culture medium following addition of IGFs. Purified oTP-1 was iodinated to a specific activity of 30-50 mCi/mg using Iodogen. Bound ^{125}I was separated from free ^{125}I by a Sephadex G-50 gel-filtration column. Rabbit antiserum raised against purified oTP-1 was diluted 1:10,000 with 50 mM Tris-HCl (pH 8.0). Samples (100 μl) were incubated with 100 μl diluted oTP-1 antiserum, 100 μl 1% BSA (in Tris-HCl), 100 μl 0.5% porcine gamma globulin (in Tris-HCl) and 25,000 cpm of ^{125}I-oTP-1 at 4°C overnight. Bound and free oTP-1 were separated by addition of 1 ml 40% polyethylene glycol (PEG 8000) followed by centrifugation at 3,600 x g for 30 min. Centrifugation was repeated after the addition of 1 ml Tris-HCl and 1 ml 40% PEG to pellets. Tubes were decanted and final pellets were subjected to gamma ray counting. The minimal detectable dose of oTP-1 was 200 pg/tube. All samples were analyzed at one time to avoid inter-assay variation and intra-assay coefficient of variation was 6.2% in the oTP-1 RIA.

Cell and Tissue Culture

Mouse embryo-derived fibroblastic cells (AKR-2B) (provided by Dr. H.L. Moses, Vanderbilt University, Nashville, TN) were grown as previously described in Materials and Methods, Chapter 2.

In separate experiments, Eagle's Minimum Essential Medium (MEM) supplemented with 1% (v/v) vitamins, 0.52% (w/v) leucine, 0.15% (w/v) methionine, 1% (v/v) nonessential amino acids, and 1% antibiotic-antimycotic solution was used for culture of conceptuses in multiwell plates (surface area of 9.62 cm^2/well). Day 13 conceptuses (five per treatment, total of 20) were collected under sterile conditions. Based on length and morphology, conceptuses were classified into three groups: spherical (<3 mm), tubular (3-8 mm), and filamentous (>8 mm). Conceptuses assigned randomly to each treatment were washed twice in MEM and then cultured in 2.5 ml MEM in the absence of IGFs, at 37°C in 5% CO_2-95% air atmosphere for 6 h. The conditioned media were collected and conceptuses were further incubated in 2.5 ml fresh MEM with the following additions: (I) phosphate-buffered saline (PBS; 0.01 M $NaPO_4$, 0.15 M NaCl; pH 7.4), (II) 100 ng/ml IGF-I, (III) 100 ng/ml IGF-II, and (IV) 50 ng/ml IGF-I and 50 ng/ml IGF-II, under the same conditions. Conditioned media were collected 24 h later, centrifuged, and stored at -20°C until analyzed.

Mitogen Assay

Mitogenic activity was monitored by measuring [^{3}H]thymidine incorporation into DNA of density-arrested, quiescent mouse embryo-derived AKR-2B cells as described in Materials and Methods, Chapter 2.

Immunoneutralization of mitogenic activity by rabbit anti-human PDGF antiserum (IgG fraction) was attempted as above, except that samples were preincubated with antibody for 1 h at room temperature prior to addition to cells.

Gel-Filtration Chromatography

ULFs from Day 14 pregnant ewes (n=3) were pooled, concentrated 10-fold by ultrafiltration using an Amicon membrane filter (cut-off=M_r 1,000; Amicon Corp., Lexington, MA), and loaded onto a Sephadex G-200 column (2.5 x 45 cm). The column was pre-equilibrated in PBS (pH 7.4) and the sample eluted in the same buffer at 4°C with a flow rate of 45 ml/h. Fractions (3 ml) were collected and 100 µl aliquots were assayed for mitogenic activity as described above. The column was calibrated with a mixture of gel-filtration M_r standards comprised of bovine thyroglobulin (670,000), bovine gamma globulin (158,000), chicken ovalbumin (44,000), horse myoglobin (17,000), and vitamin B-12 (1,350).

Statistical Analysis

Least squares analysis of variance using the General Linear Models Procedure of the Statistical Analysis System (Barr et al., 1979) was performed to examine the effect of day, status, and their interaction on IGF content and AKR-2B mitogenic activities in ULFs. A 2 x 2 factorial arrangement of treatment effects on conceptus cultures were analyzed using least squares analysis of covariance: morphology was the covariate for oTP-1 secretion in the pretreatment period, whereas oTP-1 in pretreatment media (6 h culture) was the covariate for oTP-1 production in the posttreatment period (24 h culture). Regression analysis was done to find a relationship of oTP-1 secretion with conceptus size during the first 6 h culture period. Values are shown by the least squares means ± SE unless otherwise specified.

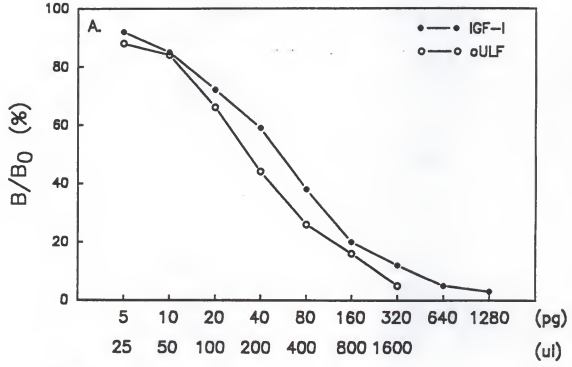
Results

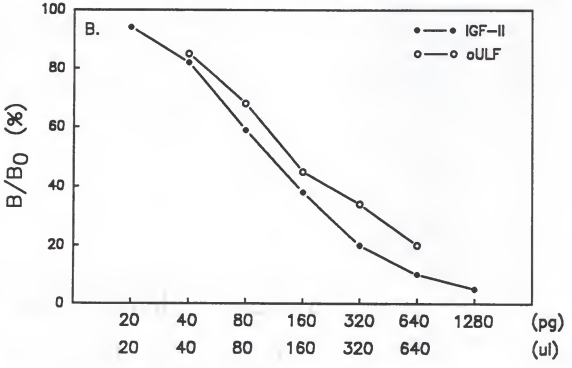
Sep-Pak^R reverse phase cartridges were used to remove IGFBPs prior to RIA analysis of ULF for IGFs (Lee et al., 1991). IGF containing proteins were eluted in 0.1% TFA in acetonitrile and were judged free of IGFBP activity from results of competitive binding assays. The RIA displacement curve for inhibition of [^{125}I]IGF-I binding to antibody by an acetonitrile eluate of a composite ULF sample is shown in Figure 3-1, panel A. This curve exhibited parallelism with rhIGF-I in a dose-dependent manner. The same ULF sample exhibited a slight deviation from the rhIGF-II reference standard with increasing doses (Figure 3-1, panel B), presumably due to interference by factor(s) specific to ULF. In this regard, IGF-II displacement curves for ovine serum, treated in the same manner, were parallel (data not shown). Since rhIGF-I had 10% crossreactivity in the IGF-II RIA (technical bulletin, Amano Corp.), IGF-II values were adjusted accordingly.

ULF IGF-I content for C and Px ewes differed ($P<0.05$) and an interaction between day and status was detected ($P<0.05$) (Figure 3-2, top panel). IGF-I content in ULF increased steadily with days of post-estrus in C, but not Px ewes. At Day 16, ULF from C and Px gilts had 0.85 ± 0.13 and 0.11 ± 0.13 ng/ml IGF-I, respectively, representing a seven-fold difference between status ($P<0.01$) in ULF IGF-I content. The corresponding IGF-II levels are presented in Figure 3-2, bottom panel. There was no difference in IGF-II content between C and Px ULFs due to effects of day; however, a difference between days within each status was detected ($P<0.01$). For

Figure 3-1. RIA displacement curves of [^{125}I]IGF-I (A) and [^{125}I]IGF-II (B).

Labeled IGF-I and IGF-II were displaced from their corresponding antibodies in a dose-dependent manner by unlabeled rhIGF-I or -II (•--•) and ovine ULF (o-o). ULF was a pool of samples from ewes at different days subjected to acidification and passage through a Sep-Pak reverse phase cartridge (Materials and Methods). B/Bo represents the ratio of cpm at each dose (B) over the cpm at zero dose (Bo) after substraction of the nonspecific cpm.


A.
IGF-I
oULF
B/B0 (%)
100
80
60
40
20
0
5 10 20 40 80 160 320 640 1280 (pg)
25 50 100 200 400 800 1600 (ul)

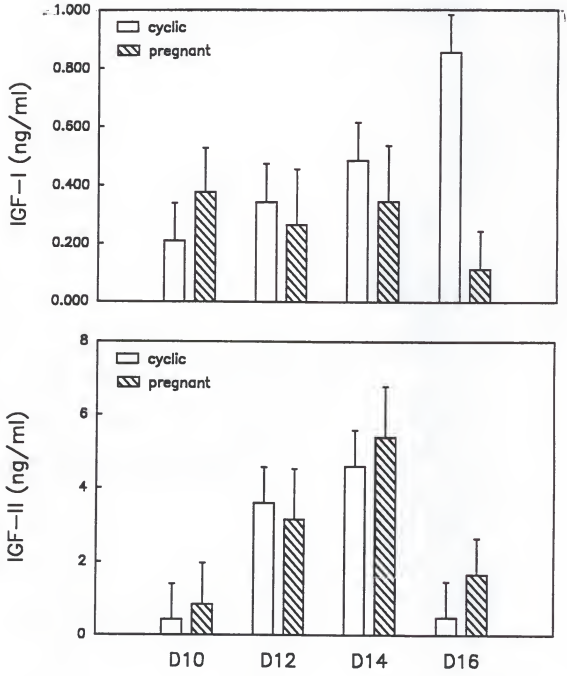

B.
IGF-II
oULF
B/B0 (%)
100
80
60
40
20
0
20 40 80 160 320 640 1280 (pg)
20 40 80 160 320 640 (ul)

Sample

Figure 3-2. The levels of IGFs in ULFs from cyclic and pregnant ewes.

(A) IGF-I levels for ULFs from C and Px ewes. ULFs were obtained at the indicated days and IGF-I measured by RIA after removal of IGF binding proteins (Materials and Methods). Each bar represents the least squares mean ± SE [n=4/day/status except Days 10 and 14 Px (n=3) and Day 12 Px (n=2)]. An effect of status (C vs Px) ($P<0.05$) and the interaction between day and status ($P<0.05$) were detected.

(B) IGF-II levels for ULFs from C and Px ewes. The same ULFs used for IGF-I RIA (panel A) were subjected to IGF-II RIA after removal of IGF binding proteins (Materials and Methods). Each bar represents the least squares mean ± SE [n=4/day/status except Days 10 and 14 Px (n=3) and Day 12 Px (n=2)]. IGF-II content was different among days ($P<0.01$), but an interaction between day and status was not found.


1.000
0.800
0.600
0.400
0.200
0.000
IGF-I (ng/ml)
cyclic
pregnant
8
6
4
2
0
IGF-II (ng/ml)
cyclic
pregnant
D10
D12
D14
D16

example, the values of IGF-II level in ULFs of Day 14 C (4.60 ± 0.98 ng/ml) and Px (5.39 ± 1.38 ng/ml) ewes are 11- and 6-fold greater than for those in ULFs for Day 10 C (0.42 ± 0.98 ng/ml) and Px (0.84 ± 1.13 ng/ml) ewes, respectively.

ULFs from C and Px ewes were also examined for presence of a mitogenic factor(s) distinct from the IGFs. Mitogenic activity was determined by monitoring stimulation of [^{3}H]thymidine incorporation into DNA of quiescent, mouse embryo-derived fibroblast cells (AKR-2B) which do not respond to IGF-I or IGF-II in the mitogen assay used (Simmen et al., 1988a). Both C and Px ULFs stimulated DNA synthesis by AKR-2B cells (Figure 3-3, panel A). A gradual increase in mitogen activity from Day 10 to 14 and a decrease on Day 16 were observed when equivalent amounts of ULF protein were examined. An effect of status (C vs. Px) was not apparent; however, differences due to day were found ($P<0.01$).

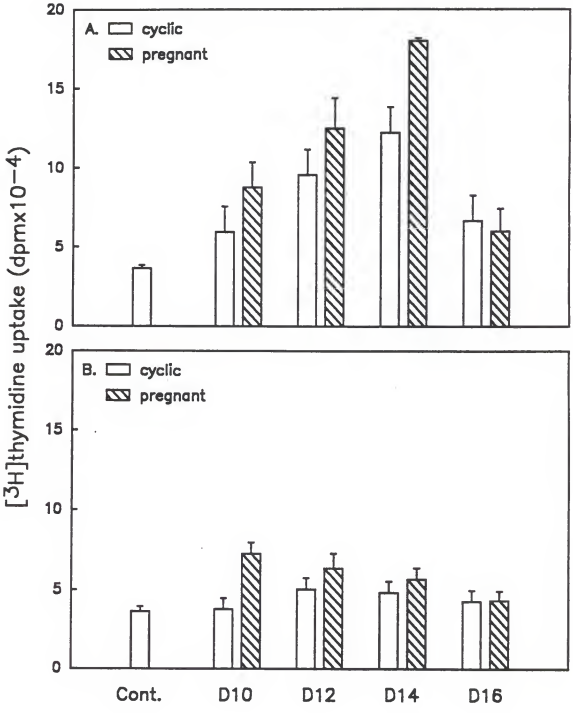
To determine whether the mitogenic activity for AKR-2B cells was susceptible to heat inactivation, mitogenic activity of crude ULF was tested after heating to 100°C for 5 min (Figure 3-3, panel B). Mitogenic activity in all ULFs of C ewes was uniformly lowered by heat-treatment. In contrast, mitogenic activity in the ULFs of Px ewes at the various days was differentially affected by heating. Mitogenic activity of Day 10 Px ULF was unaffected; however, ULFs from Day 12 Px and Day 14 Px ewes had 2- and 3-fold reductions in mitogenic activity after heat treatment ($P<0.05$).

ULF from Day 14 Px ewes was characterized further since it exhibited the highest relative mitogenic activity. Pooled Day 14 ULF proteins were size-fractionated by Sephadex G-200 gel-filtration chromatography (Figure 3-4). Column fractions were

Figure 3-3. Mitogenic activities of cyclic and pregnant ewe ULFs before and after heat-treatment.

(A) Mitogenic activities of crude ULFs from C and Px ewes. Equal amounts of ULF proteins (25 μg/ewe) in 100 μl final volume adjusted with PBS were tested in triplicate for stimulation of [^{3}H]thymidine incorporation into DNA of AKR-2B cells relative to the control treatment (PBS). Each bar represents the least squares mean ± SE of activity of individual ULFs [n=3/day/status except Day 12 Px (n=2) and Day 16 Px (n=4)]. There were no differences between C and Px ewe ULFs, but differences among the days were found ($P<0.01$).

(B) Mitogenic activity of C and Px ewe ULFs after heat-treatment. ULFs (25μg protein/ewe) were placed in boiling water for 5 min, adjusted to 100 μl final volume per well with PBS and monitored for mitogenic activity in triplicate using AKR-2B cells. Each bar represents the least squares mean ± SE [n=3/day/status except Day 12 Px (n=2) and Day 16 Px (n=4)] for each day. Differences among days were not found, but an effect of status was detected ($P<0.05$).


A. cyclic
pregnant
B. cyclic
pregnant
[3H]thymidine uptake (dpmx10−4)
0
5
10
15
20
Cont.
D10
D12
D14
D16

then monitored by mitogen assay. Two peaks of mitogenic activity were observed: one eluted in the column void volume with an activity 50-fold over basal (buffer only) while the other eluted with an apparent M_r of 30,000 and an activity 16-fold over basal. Immunoneutralization of crude ULF (Day 14 Px) and pooled G-200 fractions (#52-56) was attempted using rabbit antiserum to human PDGF (IgG fraction) (Figure 3-5) since the second eluting peak had a native M_r similar to that of PDGF (Ross et al., 1986) (Figure 3-4). Normal rabbit serum (NRS;IgG fraction) constituted the control treatment. PDGF IgG had no effect on the M_r of 30,000 mitogenic activity; however, the activity of crude ULF was unexpectedly stimulated 180% by addition of PDGF antibody (Figure 3-5).

The in vitro effects of IGFs on oTP-1 secretion were next examined (Table 3-1). Conceptuses at Day 13 were utilized since this developmental time point represents rapid initiation of synthesis and secretion of oTP-1 (Nephew et al., 1991) and because conceptuses are large enough to culture individually for collection of culture medium. Due to the limitation of numbers of embryos available, only single concentrations of IGF-I and IGF-II (individually and in combination) could be tested. A final concentration of 100 ng/ml IGF was arbitrarily chosen for this first experiment. Ovine TP-1 secretion during PRE incubation period (culture without IGFs) was correlated (PRE $r^2=0.45$; $P<0.01$) to conceptus size, showing a positive linear relationship ($Y = 4.20X - 2.42$) of oTP-1 secretion (Y) with conceptus size (X) (Figure 3-6). Ovine TP-1 secretion during the POST period (culture with IGFs) was also correlated ($r^2=0.48$; $P<0.01$) with PRE oTP-1 secretion, regardless of treatment.

Figure 3-4. Gel-filtration chromatography of ULF from Day 14 pregnant ewes.

Equivalent volumes of Day 14 Px ewe ULFs (n=3) were pooled, concentrated and 3.7 mg total ULF protein was applied to a Sephadex G-200 column preequilibrated with PBS (pH 7.4). The absorbance profile for eluted proteins at 280 nm is indicated by (o-o). Mitogenic activity (•-•) was determined by testing aliquots (100 μl), in triplicate, of fractions (3 ml) on AKR-2B cells. Arrows indicate the elution positions of molecular weight markers: Vo (void volume, bovine thyroglobulin, 670,000), 158,000 (bovine gamma globulin), 44,000 (chicken ovalbumin), 17,000 (horse myoglobin) and 1,350 (Vit. B-12). Fractions 52-56 were pooled and concentrated for further analysis (see Figure 3-5).

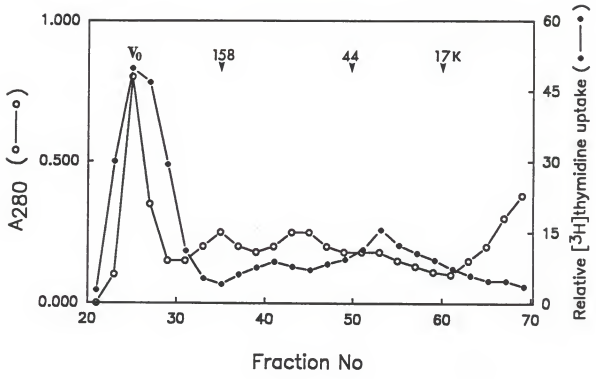

1.000
0.500
0.000
A280 (o—o)
60
45
30
15
0
Relative [3H]thymidine uptake (•—•)
V0
158
44
17K
20
30
40
50
60
70
Fraction No

Figure 3-5. Stimulation by ULF proteins of [^{3}H]thymidine incorporation into AKR-2B cellular DNA: effects of PDGF IgG and normal rabbit serum IgG.

Unfractionated (shown as oULF) and fractionated (shown as Factor; pooled G-200 column fractions 52-56) D14 Px ULF were preincubated with rabbit anti-human PDGF antiserum or normal rabbit serum (IgG fractions; 100 μg) for 1 h at room temperature. Mitogenic activities of those mixture on AKR-2B cells were tested and compared to those of PBS-treated control cells. Each bar represents the mean ± SEM of triplicate wells.

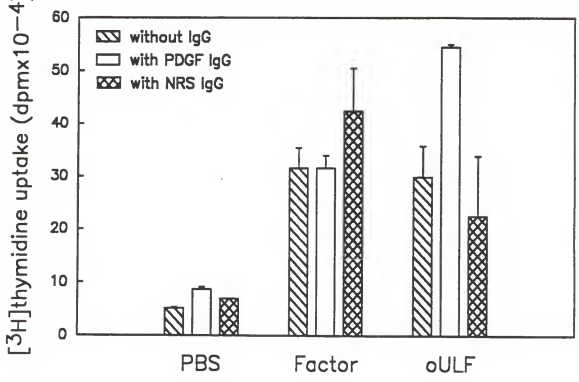

[3H]thymidine uptake (dpmx10−4)
60
50
40
30
20
10
0
without IgG
with PDGF IgG
with NRS IgG
PBS
Factor
oULF

Table 3-1. Effects of IGFs on oTP-1 secretion in vitro.[a]

Embryo		oTP-1 (ng/ml/6 h)[b]	
Treatment	Morphology[c]	PRE[d]	POST[e]
I) no IGF	S,T,T,T,F	20.6 ± 30.0	17.3 ± 11.1
II) IGF-I (100 ng/ml)	S,T,T,F,F	76.4 ± 30.0	9.9 ± 11.2
III) IGF-II (100 ng/ml)	S,T,F,F,F	49.8 ± 30.0	13.0 ± 10.7
IV) IGF-I/-II (50, 50 ng/ml)	S,T,T,F,F	43.0 ± 30.0	48.9 ± 10.8*

[a] Day 13 conceptuses (n=5 per treatment) were cultured without IGFs for 6 h (PRE) followed by change to medium containing indicated amounts of IGFs at 37°C for 24 h (POST). Amounts of oTP-1 in conceptus conditioned culture media were measured by RIA (Material and Methods) and expressed by the same period of incubation time (6 h).

[b] Values are least squares means ± SE.

[c] Morphology of individual conceptuses was classified as S (spherical: <3 mm), T (tubular: 3-8 mm), and F (filamentous: >8 mm).

[d] Morphology was the covariate.

[e] PRE oTP-1 was the covariate.

* $P<0.01$

When PRE oTP-1 secretion was used as a covariate in the analysis, a treatment effect was detected ($P<0.01$): oTP-1 secretion by the IGF-I + IGF-II treated conceptuses was 3-fold greater than for conceptuses in other treatment groups. However, addition of either IGF-I or IGF-II alone did not stimulate oTP-1 secretion over basal levels. In a separate experiment, based on the interferon properties of oTP-1 (Farin et al., 1989; Guillomot et al., 1990), antiviral activity in culture media was measured to corroborate results of the oTP-1 RIA. Antiviral activity in individual media samples was highly correlated with oTP-1 levels (data not shown). RIA analysis of PRE media for the presence of IGF-I and IGF-II did not detect

immunoreactive IGF peptides, possibly reflecting the short period of time (6 h) used for the PRE incubations and/or negligible release of IGFs by the Day 13 conceptuses. However, culture of Days 14 and 16 conceptuses for longer periods of time, followed by IGF RIA of conditioned serum-free media, indicated a preferential (30-fold greater) release of IGF-II over IGF-I (Figure 3-7).

Discussion

Sheep conceptuses undergo growth as well as morphological differentiation (transitions from spherical to tubular to filamentous forms) at Days 13-14, immediately prior to attachment to the uterine epithelium (Boshier, 1969). The molecular basis for programmed conceptus development is obscure, although studies of sheep conceptus secretory proteins have identified oTP-1 as a product of a conceptus gene whose expression is induced specifically during this period (Farin et al., 1990). The objective of the present study was to characterize the relative levels of insulin-like and other growth factors in ovine ULFs during the peri-attachment period as an initial step to defining the possible physiological linkages of ULF growth factors and coordinate conceptus and endometrial tissue growth and differentiation. Results demonstrated the presence in the ovine uterine lumen of at least four distinct mitogenic factors: IGF-I, IGF-II, and the two peaks of mitogenic activity identified using AKR-2B indicator cells.

Figure 3-6. A linear relationship of oTP-1 secretion with conceptus size.

Day 13 conceptuses were collected under sterile condition, washed twice in MEM, and cultured singly in 2.5 ml serum-free MEM at 37°C in 5% CO_2 incubator for 6 h. Ovine TP-1 levels in conditioned media were measured by RIA, as described in Materials and Methods. Each filled circle represents oTP-1 amount secreted from individual conceptus and a regression line was drawn. The secretion of oTP-1 (Y) was positively correlated to conceptus size (X) in a linear fashion (Y = 4.20X - 2.42).

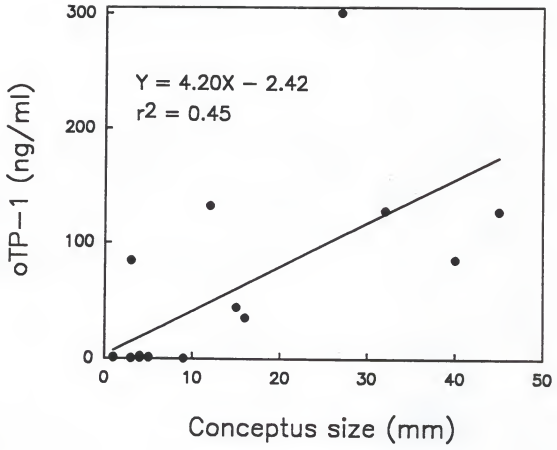
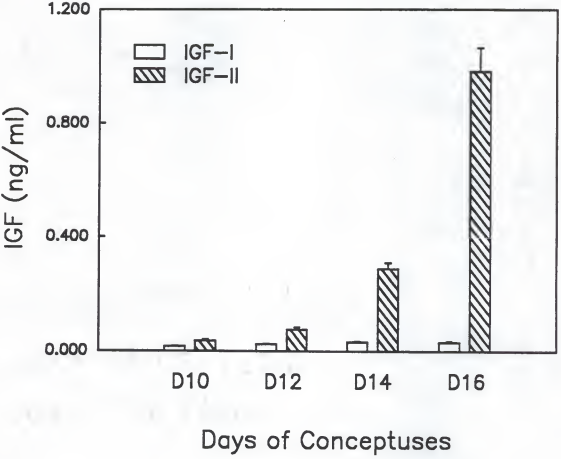

Y = 4.20X − 2.42
r2 = 0.45
oTP−1 (ng/ml)
300
200
100
0
0
10
20
30
40
50
Conceptus size (mm)

Figure 3-7. IGF-I and IGF-II content in conceptus culture medium.

Days 10-16 conceptuses were cultured singly at 37°C for 24 h in serum-free medium. Equal volumes of culture medium at each developmental stage were pooled and subjected for RIA after removing binding proteins (Materials and Methods). Open bars represent IGF-I content and hatched bars IGF-II content, shown as the mean ± SEM (n=3).


1.200
0.800
0.400
0.000
IGF (ng/ml)
IGF-I
IGF-II
D10
D12
D14
D16
Days of Conceptuses

Uterine IGF synthesis and secretion are regulated by stage of gestation (for IGF-I and IGF-II) (Letcher et al., 1989a; Simmen et al., 1990, 1992) and by the steroid hormones estrogen and progesterone (for IGF-I) (Murphy et al., 1987b). During peri-implantation development in the pig, initiation of estrogen production by conceptuses on Day 12 (Geisert et al., 1982b) is coincident with maximal IGF-I in ULF and peak endometrial IGF-I mRNA abundance (Letcher et al., 1989a; Simmen et al., 1992). Quantitation of IGF-II in porcine or ovine ULF has not been reported. However, expression of IGF-II mRNA, which is regulated differentially from that of IGF-I, was demonstrated in uterine endometrium of gilts subjected to different steroidal treatments (Simmen et al., 1990) and during the estrous cycle and early pregnancy (Simmen et al., 1992). In the present study, there was a gradual rise in ULF IGF-I content (Days 10 to 16; C ewes) and ULF IGF-II content (Days 10 to 14; C and Px ewes) during diestrus (Figure 3-2). In pregnant ewes, these changes correspond to increasing concentrations of peripheral progesterone during the luteal phase (Hauger et al., 1977). In cyclic ewes, temporal changes in ULF IGF-II were similar to those for Px ewes. However, ULF IGF-I increased further between Days 14 and 16 for C ewes, unlike the decrease for Px ewes during the same period. These results suggest the possibility of differential stimulation of ULF IGF-I by ovarian follicular estrogen during proestrus of C, but not Px ewes (Hansel et al., 1973). Alternatively, the decreased level of IGF-I in Day 16 Px ULF compared to Day 16 C ULF may reflect conceptus uptake of this growth factor. Physiological functions for uterine IGFs have not been clearly defined for any animal model.

However, endometrial and myometrial cells express cell-surface receptors for IGFs (Rutanen et al., 1988; Ghahary and Murphy., 1989; Hofig et al., 1991a) and endometrial stromal and epithelial cells in culture respond to exogenous IGFs by increased mitogenesis (Simmen et al., 1988c; Chapter 4).

That ULF IGFs originate at least in part from the uterus is suggested by the presence of mRNAs encoding both peptides in sheep endometrium (F.A. Simmen, unpublished data) and bovine endometrium (Geisert et al., 1991). The conceptus may also provide a source of IGFs since IGF proteins (in particular IGF-II) were detected in conceptus culture media corresponding to the later filamentous stages (Figure 3-7). In ovine ULFs, IGF-II was quantitatively greater than IGF-I (Figure 3-2). In contrast to different temporal patterns for IGF-I between C and Px ewes, uterine luminal IGF-II content was greatest on Day 14 and then declined on Day 16 in both C and Px ewes. This parallels closely the expression of an IGF-binding protein (IGFBP)-1 in early Px sheep endometrium (Waites et al., 1990). Immunologically, IGFBP-1 protein is first detected in the luminal epithelium of sheep endometrium at Day 10, peaks at Day 14, and subsequently decline by Day 16 of pregnancy (Waites et al., 1990). Data pertaining to IGFBP-1 in ULF as a function of gestation are not available. Similarly, the presence or absence of other IGFBPs (e.g., IGFBP-2 and IGFBP-3) in ULF of C and Px ewes remains to be clarified.

Temporal variation in total AKR-2B mitogenic activity in ULF mimicked the developmental profile for IGF-II in C and Px ewes and IGF-I in Px ewes. Highest activity was on Day 14 with a decline noted for Day 16 for both C and Px ewes. The

mitogenic activity of ovine ULF is extremely potent since as little as 1 μg of total protein stimulated DNA synthesis above control. The M_r of 30,000 for the second eluting peak of AKR-2B activity suggested a possible correspondence to platelet-derived growth factor, a known AKR-2B mitogen (Leof et al., 1986). However, attempts to immunoneutralize partially purified 30 kDa mitogenic activity with PDGF antibody were unsuccessful. Addition of PDGF IgG to crude ULF, however, unexpectedly and consistently stimulated [^{3}H] thymidine uptake by AKR-2B cells above the control of NRS IgG treated samples. The basis for this observation is unclear.

The high M_r activity eluting in the column void volume may correspond to a high M_r endothelial mitogen(s) described by Reynolds et al. (1990a). They reported the secretion in vitro of endothelial cell mitogen(s) from ovine endometrium obtained during early pregnancy. Their factor(s) exhibits a large M_r (>100,000) and an acidic pI value and binds to heparin-Sepharose. Alternatively, the high M_r activity observed in the present study may represent an aggregate of proteins which eluted early from the column. Furthermore, this activity may correspond to specific complexes of mitogen and one or more binding proteins. Porcine ULF, like ovine ULF, contains an AKR-2B active mitogen (denoted ULFM until final identification; Chapter 2). Levels of this mitogen vary with day of the estrous cycle or pregnancy (Simmen et al., 1989a). However, the ovine and porcine ULF factors differ markedly in native M_r (30,000 vs. 4,800) and in degree of inactivation by heat (sensitive vs. insensitive) (Chapters 2 and 3) which suggest the absence or diminution of ULFM in ovine ULF.

Further purification and characterization is required to clarify the nature of the AKR-2B active ULF factors and to examine their postulated roles in uterine and conceptus development (Simmen et al., 1989a).

Elongating conceptuses synthesize an array of proteins including oTP-1, the signal for maternal recognition of pregnancy in sheep (Godkin et al., 1982b). The binding of oTP-1 to endometrial interferon receptors (Godkin et al., 1984a; Hansen et al., 1989; Knickerbocker & Niswender, 1989) results in the induction of synthesis and secretion of several endometrial proteins (Godkin et al., 1984a; Vallet et al., 1987; Sharif et al., 1989; Ashworth & Bazer, 1989). Although the identities and physiological roles of the oTP-1-induced proteins are not well defined, they could conceivably serve to promote conceptus development, endometrial differentiation and/or luteal maintenance (Vallet et al., 1987). In particular, oTP-1 induction of endometrial growth regulatory molecules provides a mechanism whereby the conceptus might regulate endometrial growth and/or differentiation. Although an attractive hypothesis, this is not supported by the observed lack of differences in mitogen levels in ULFs from Px vs C ewes. Rather, results of the present study suggest that differences in sheep ULF growth factor activity among days largely reflects programmed and/or hormonally regulated temporal changes in uterine activity independent of the conceptus.

Ovine TP-1 secretion during PRE-treatment period was positively correlated to conceptus size and morphology. This agrees with the results of Nephew et al. (1991) that more oTP-1 was recovered from uterine flushings of ewes having

developmentally advanced conceptuses. By use of in situ hybridization, Farin et al. (1989) localized oTP-1 mRNA in conceptus trophectoderm, with peak mRNA abundance noted at Day 13 of pregnancy. In a separate study, oTP-1 was detected on Day 11, expressed maximally at Day 14 and decreased by Day 16 (Guillomot et al., 1990); these changes temporally coincide with ULF IGF-II and AKR-2B mitogen levels of the present study. Concomitantly, oTP-1 is present at low levels (1-2 ng/ml) in ULFs of Days 10-12 Px ewes and at high levels (3-4 μg/ml) in ULFs of Days 14-16 Px ewes (T.L. Ott and F.W. Bazer, unpublished data).

Results in vitro suggest a synergism of IGF-I and IGF-II to stimulate or at least maintain oTP-1 secretion and neither IGF-I or IGF-II alone altered oTP-1 secretion over basal levels. IGFs are known regulators of protein synthesis and secretion in other systems (Sara & Hall, 1990; Humbel, 1990) and also suggested as survival factors for embryonic cells (Heath and Rees, 1985). For these factors to exert a direct effect on conceptus secretion requires the simultaneous expression of IGF receptors on trophectoderm. Data regarding presence of IGF receptors on sheep conceptuses are not available, whereas pig trophoderm cells at similar stages of conceptus development exhibit IGF receptors (Corps et al., 1990) and pig conceptuses respond in vitro to exogenous IGF-I via increased aromatase activity (Hofig et al., 1991b). Ashworth and Bazer (1989) investigated the effect of endometrial proteins on oTP-1 production in vitro. Their results demonstrated increased concentrations of oTP-1 in culture media when endometrium and conceptus tissues were co-cultured, suggesting a requirement for physical interaction

between conceptus and endometrium for induction of oTP-1 production.

In summary, the presence of physiological levels of IGF-I, IGF-II and additional, as yet unidentified, mitogenic factors in ULFs of cyclic and pregnant ewe has been documented. The temporal changes in abundance of uterine luminal IGF-II during the periattachment period, coupled with the apparent expression of IGFs in endometrial and conceptus tissues, suggest important regulatory functions for these proteins at the conceptus-maternal interface. Furthermore, the possible in vivo linkages of IGFs and conceptus production of oTP-1 in sheep (this study) or estrogens in pigs (Hofig et al., 1991b), as suggested in vitro, may indicate a general role for the insulin-like growth factors in maternal recognition of pregnancy.

CHAPTER 4
IN VITRO EFFECTS OF PEPTIDE GROWTH FACTORS ON UTERINE EPITHELIAL CELL GROWTH AND DIFFERENTIATION

Introduction

The uterus is an important site of synthesis and secretion of regulatory proteins during implantation and late pregnancy (Roberts and Bazer, 1988; Tabibzadeh, 1991; Simmen and Simmen, 1991). Polypeptide growth factors that mediate tissue growth and differentiation are important constituents of uterine tissues and secretions. These include the insulin-like growth factors (IGFs) -I and -II (Murphy et al., 1987b; Ghahary et al., 1990; Simmen et al., 1990, 1992), epidermal growth factor (EGF) (Tomooka et al., 1986; Huet-Hudson et al., 1990; Hofmann et al., 1991), acidic and basic fibroblast growth factors (a/bFGFs) (Brigstock et al., 1990), transforming growth factors (TGFs) -α and -ßs (Han et al., 1987; Tamada et al., 1990), and colony-stimulating factors (Pollard, 1990; Kauma et al., 1991). Results from in vivo and in vitro studies support a role for these proteins in the regulation of fetal-placental growth, as well as uterine growth and differentiation (Tomooka et al., 1986; Paria and Dey, 1990; Simmen and Simmen, 1990, 1991).

In vitro cell culture systems are useful for characterizing the actions of peptide growth factors (Riss and Sirbasku, 1987; Paria and Dey, 1990; Mouzon and Kahn, 1991; Irwin et al., 1991). However, the relative paucity of continuous uterine

epithelial cell lines limits the elucidation of their biological responses to these regulatory proteins. The establishment of cell lines, transformed with temperature-sensitive simian virus 40 (SV40) and exhibiting the characteristics of the original cell population but with an unlimited life span, has now allowed studies to monitor the response of cells to exogenous stimulatory and inhibitory factors (Chou, 1989; Li et al., 1989; Fliss et al., 1991b).

Peptide growth factors can interact with target cells via endocrine, paracrine, and autocrine routes (Sporn and Roberts, 1985; Sara and Hall, 1990; Tabibzadeh, 1991; Simmen and Simmen, 1990, 1991). In the uterus, growth factors produced by the stroma are thought to mediate growth and differentiation of luminal and glandular epithelium as well as the stroma (Murphy and Ghahary, 1990; Tabibzadeh, 1991; Donjacour and Cunha, 1991). However, it is at present difficult to evaluate the validity of this model due to the lack of information concerning the specific growth requirements of uterine epithelial cells. The present study has, therefore, evaluated the differential effects of peptide growth factors on proliferation, and on protein synthesis and secretion of a rabbit uterine epithelial cell line previously established by infection with a temperature-sensitive transforming virus (Li et al., 1989).

Materials and Methods

Materials

Alpha-modified minimum essential medium (MEM), leucine-free MEM, phenol red-free MEM, and fetal bovine serum (FBS) were purchased from GIBCO (Grand

Island, NY). Antimycotic-antibiotic, trypsin-EDTA, and phosphate buffered saline (PBS) solutions, and monoclonal antibodies to bovine cytokeratin and porcine vimentin were from Sigma Chemical Co. (St. Louis, MO). Biomeda histoscan staining kit was purchased from Biomeda Corp. (Foster City, CA) and culture chamber slides were from Nunc (Naperville, IL). Recombinant human growth factors [IGF-I, IGF-II, EGF, acidic FGF (aFGF), and TGF-β1] were obtained from Amgen Biologicals (Thousand Oaks, CA). Bovine insulin and recombinant human basic FGF (bFGF) were from Upstate Biotechnology, Inc. (Lake Placid, NY). [Methyl-^{3}H]thymidine (5 Ci/mmol), L-[4,5-^{3}H]leucine (142 Ci/mmol), and a nick-translation kit were purchased from Amersham Corp. (Arlington Heights, IL). Multiwell plates (surface area of 2.0 cm^2/well) and tissue culture dishes were from Becton-Dickinson (Lincoln Park, NJ) and Corning Glass Works (Corning, NY), respectively. Disuccinimidyl suberate (DSS) was from Pierce Chemical Co. (Rockford, IL) and Ampholines (pH 3.5-10) were from Pharmacia LKB Biotechnology (Piscataway, NJ). Nitrocellulose and nylon (Biotrans) membranes were manufactured by Schleicher and Schuell, Inc. (Keene, NH) and ICN (Irvine, CA), respectively. Geneclean II kit was from Bio 101 (La Jolla, CA).

Cell Cultures

The rabbit endometrial epithelial cell line, HRE-H9, was established by infection of dispersed uterine epithelial cells with an origin-defective, temperature-sensitive mutant of SV40 at 33°C (Li et al., 1989). The clonal epithelial cell line obtained reverts to a nontransformed state at 37-40°C. HRE-H9 cells were grown in MEM

(pH 7.4) supplemented with 5% heat-inactivated FBS (MEM-FBS) and 1% antimycotic-antibiotic solution. Cells were incubated at either 33 or 37°C in a humidified 5% CO_2 in air atmosphere. Cells representing the sixth to twentieth passages were subcultured using trypsin-EDTA solution and used in the present studies.

Immunohistochemical Staining of H9 Cells

Cells seeded in culture chamber slides were grown to 80-90% confluence in MEM-FBS at 37°C in 5% CO_2-forced incubator. Cells were washed with warmed PBS followed by cold PBS three times each. Cells were fixed with cold methanol or methanol-acetone (1:1) mixture for 10 min. Following PBS wash, endogenous peroxidase was blocked by incubation with 3% H_2O_2 for 10 min. Cells were rinsed twice with PBS and incubated with tissue conditioner and 10% normal goat serum (NGS) to block non-specific background for 20 min of incubation without rinsing. Monoclonal antibodies to bovine cytokeratin and porcine vimentin were applied for the primary antibody at a dilution of 1:50. The primary antibodies were applied and incubated for 2 h in a moist chamber at 37°C. After cells were rinsed twice with PBS (final rinse with 2% NGS), the anti-mouse second antibody conjugated to biotin was added to cells which were rinsed twice. After incubating with peroxidase reagent for 30 min, cells were again rinsed. Four drops of chromagen (3-amino-9-ethylcarbazole) solution was added and incubated for 5 min to resolve or visualize the enzyme substrate complex. One drop of hematoxylin used as a counterstain was added to stain nuclei and incubated for 1 min. Cells were rinsed with water for 5

min and coverslip was put onto the slide with glycerol gelatin. Photos were taken on a Nikon Diaphot-2 with Ektar 25 film by Kodak.

Mitogen Assay

Mitogenic effects of exogenous peptide growth factors were monitored via the incorporation of [^{3}H]thymidine into DNA of serum-deprived, density-arrested HRE-H9 cells, as previously described in Materials and Methods, Chapter 2, with minor modifications. Briefly, cells (7.5 x 10^4/ml/well) were seeded in 24-well plates and grown in 5% MEM-FBS at 33 or 37°C. When confluent, cells were refed with fresh MEM containing 1% FBS and further incubated for 48 h to ensure quiescence. Cells were washed twice with serum-free medium and growth factors were added to triplicate wells per treatment in serum-free MEM. After incubation for an additional 20 h, cells were pulse-labelled with [^{3}H]thymidine (1 μCi/ml) for 4 h. Monolayers were rinsed with PBS, cellular DNA was precipitated with trichloroacetic acid (TCA), and incorporated radioactivity was measured by liquid scintillation counting.

Incorporation of Radiolabeled Leucine

Effects of growth factors on protein synthesis were monitored following the procedure of Mans and Novelli (Mans and Novelli, 1961) with minor modifications. Cells (7.5 x 10^4/ml/well) were grown to 80-90% confluence at 37°C in leucine-free, MEM-FBS and starved in fresh serum-free, leucine-free MEM for 24 h. The cells were washed with serum-free, leucine-free MEM twice and growth factors and [^{3}H]leucine (10 μCi/ml) were added to wells in triplicate. After incubation for 24 h, conditioned media (CM) were collected by centrifugation at 4,800 x g, 4°C for 30

min and cell monolayers solubilized in 0.3 M NaOH. Twenty microliters of CM and cell lysates (CL) were spotted onto Whatman filters pretreated with 20% TCA. The filters were air-dried and sequentially washed twice in 10% TCA for 5 min at 65°C and once in 95% ethanol for 5 min at RT. Radioactivity in TCA-precipitated proteins was measured by liquid scintillation counting.

Two-Dimensional SDS-PAGE and Fluorography

Two-dimensional (2D) SDS-PAGE and fluorography were conducted according to procedures described by Roberts et al. (1984). Cells (1 x 10^6/60 mm dish) grown to confluence in leucine-free, MEM-FBS were starved for 24 h in fresh serum-free, leucine-free MEM and growth factors and [^{3}H]leucine (50 μCi/ml) were added. After incubation for 24 h, CMs were pooled and CLs were prepared by a brief sonication. Both CM and CL were centrifuged at 4,800 x g for 30 min. Supernatants were dialyzed (M_r 3,000 cutoff) against 10 mM Tris (pH 7.4) twice and then against water. Dialyzed samples (100,000 cpm of CM and 150,000 cpm of CL) were lyophilized and dissolved in isoelectric focusing (IEF) sample buffer [9.3 M urea, 2% Triton X-100, 2% Ampholines (pH 3.5-10), and 0.5% dithiothreitol in 5 mM potassium carbonate]. IEF was carried out sequentially at 75 volts for 30 min, 150 volts for 2 h, and 300 volts for 16 h. Proteins were separated in the second dimension by 10% SDS-PAGE under reducing conditions. Gels were stained with Coomassie Brilliant Blue, washed in tap water for 30 min, treated with 1 M sodium salicylate for 30 min to enhance sensitivity of fluorography, dried, and exposed to X-ray films. The M_r standards used were phosphorylase b (97,000), bovine serum

albumin (BSA) (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,000), and lysozyme (14,000).

Northern Blot Analysis

Total cellular RNA was isolated from HRE-H9 cells by the guanidine thiocyanate method following the procedure of Puissant and Houdebine (1990) with minor modifications for cells. RNA concentrations were estimated from optical absorbance at 260 nm (absorbance unit = 33 μg RNA/ml). A 430 base pair (bp) rabbit uteroglobin cDNA insert (generously provided by Dr. DW Bullock) was purified using Geneclean II kit after restriction-endonuclease (PstI) digestion and 1% agarose gel electrophoresis, and used as a probe following nick-translation.

Forty microgram of total RNA was separated in a 1.5% agarose gel containing 2.2 M formaldehyde following the procedure of Thomas (1980). After electrophoresis, RNA was transferred to a nylon membrane and baked for 2 h at 80°C. Complementary DNA probe with a specific activity was prepared using a nick-translation kit. After prehybridization for 2 h at 42°C in prehybridization solution containing 2X SSC (1X = 150 mM NaCl and 15 mM sodium citrate, pH 7.0), 50% formamide, 5X Denhardt's solution [1X = 0.02% (w/v) for each of Ficoll, polyvinyl-pyrrolidone, and BSA], 40 mM sodium phosphate (pH 6.5), 0.1% SDS, and yeast RNA (250 μg/ml), the membranes were hybridized with 3-5 x 10^6 cpm cDNA/ml in fresh prehybridization solution containing 1X Denhardt's solution at 42°C overnight. The filters were washed with 2X SSC-0.1% SDS at room temperature for 30-60 min followed by 0.1X SSC-0.1% SDS at 60°C for 15-60 min and exposed to X-Omat RP

films (Eastman Kodak, Rochester, NY) with intensifying screens at -80°C. Sizes of mRNA was calculated on the assumption that lengths of 28S and 18S rRNAs, as visualized by ethidium bromide staining, are 5 Kbp and 2 Kbp, respectively.

Affinity Cross-Linking

IGF receptors on HRE-H9 cells were identified following the method of Ritvos et al. (1988b). Cells (1 x 10^6/60 mm dish for each treatment) were grown to confluence at 33°C or 37°C in MEM-FBS, incubated further in serum-free MEM for 24 h, and washed twice with ice-cold PBS containing 0.1% BSA (PBS-BSA). Using Iodogen, IGFs were iodinated to a specific activity of ~300 μCi/μg as previously described (Lee et al., 1991). Either 500,000 cpm (0.2 nM) of [^{125}I]IGF-I or [^{125}I]IGF-II in 2.0 ml of PBS-BSA was incubated with cells at 4°C for 16 h and cells were rinsed twice with ice-cold PBS-BSA to terminate the reaction. DSS, freshly dissolved in dimethylsulfoxide, was added in 1.5 ml PBS-BSA to give a final concentration of 0.5 mM. Cross-linking was carried out for 30 min at RT and quenched by addition of 3 vol of 10 mM Tris (pH 7.4) and standing for 5 min. Cells were collected and solubilized in 350 μl of 60 mM Tris (pH 7.4) containing 2 % SDS. Cell lysates were mixed with sample buffer [62.5 mM Tris (pH 6.8), 5% SDS, 10% sucrose, 0.02% bromophenol blue, and 5% β-mercaptoethanol], boiled for 3 min, and electrophoresed in 10% SDS-PAGE following the procedure of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue, dried, and subjected to autoradiography using X-Omat AR film. The SDS-PAGE M_r standards were myosin

(200,000), ß-galactosidase (116,000), phosphorylase b (97,000), BSA (66,000), and ovalbumin (45,000).

Ligand Blot Analysis of IGF Binding Proteins

Cells (1 x 10^6/60 mm dish) were grown to confluence at 33°C or 37°C in MEM-FBS, and incubated in serum-free MEM for 24 h. Medium was removed, cells were rinsed with serum-free MEM and further incubated in the same medium for 24 h (CM). CMs and CLs were dialyzed and their protein contents determined by the Bradford protein assay (Bradford, 1976). DNA amounts in CLs were also measured by fluorometric DNA assay (Labarca and Paigen, 1980). Lyophilized proteins (400 µg) were reconstituted in sample buffer without ß-mercaptoethanol, boiled for 3 min, and subjected to 12.5% SDS-PAGE under non-reducing conditions. Ligand blotting was performed following the procedure of Hossenlopp et al. (1986) as modified by Lee et al. (1991). Proteins were electrotransferred to a nitrocellulose membrane (0.2 µm) for 5 h at 200 mA. The membrane was dried and incubated in Tris-buffered saline [TBS; 10 mM Tris (pH 7.4), 0.15 M NaCl, 0.05% NaN_3] containing 1% nonfat dry milk for 1 h at RT. After a rinse in TBS-0.1% Tween-20 for 10 min at RT, the membrane was incubated with [^{125}I]IGF-II (200,000 cpm/ml) in TBS containing 0.1% Tween-20 + 1% BSA, for 6 h at RT. The membrane was washed in TBS-0.1% Tween-20 twice followed by a rinse in TBS, air-dried, and subjected to autoradiography. Bovine neonatal serum was co-electrophoresed as a positive control for presence of IGFBPs (Lee et al., 1991).

Statistical Analysis

Analysis of variance was performed using the General Linear Models Procedures of the Statistical Analysis System (Barr et al., 1979). The effects of temperature, treatment, and interaction of temperature x treatment on DNA synthesis by IGF-I, and the effects of peptide growth factors on DNA and protein synthesis and protein secretion were examined. Regression analyses were done to find growth factor amounts which give half-maximal stimulation of DNA synthesis of HRE-H9 cells. Data are expressed as the means ± SE unless otherwise specified. Significance of differences between means was evaluated by use of the Student's t-test or Duncan's multiple range test (Steel and Torrie, 1980).

Results

Immunohistochemical staining study demonstrated the epithelial nature of HRE-H9 cells, which was indicated by positive staining with an anti-cytokeratin antibody and by negative staining with an anti-vimentin antibody (A and B, respectively, in Figure 4-1). In contrast, as a negative control, mouse embryo-derived AKR-2B fibroblasts were stained, resulting in an opposite staining (C and D in Figure 4-1).

The HRE-H9 cell line exhibits reversible growth properties at permissive (33°C) and non-permissive (40°C) temperatures (Li et al., 1989). Cells in the transformed and nontransformed states were evaluated for responses to exogenous growth factors by monitoring incorporation of [^{3}H]thymidine into cellular DNA (Table 4-1). The

Figure 4-1. The epithelial nature of HRE-H9 cells by immunohistochemical staining.

HRE-H9 (A and B) and AKR-2B (C and D) cells grown at 37°C were fixed with methanol. Cells were incubated with monoclonal antibodies to bovine cytokeratin (A and D) and porcine vimentin (B and C) at a dilution of 1:50 for 2 h in a moist chamber at 37°C and stained as described in Materials and Methods. Positive staining of HRE-H9 cells (A) with anti-cytokeratin antibody demonstrates that H9 cells are endometrial epithelial cells.

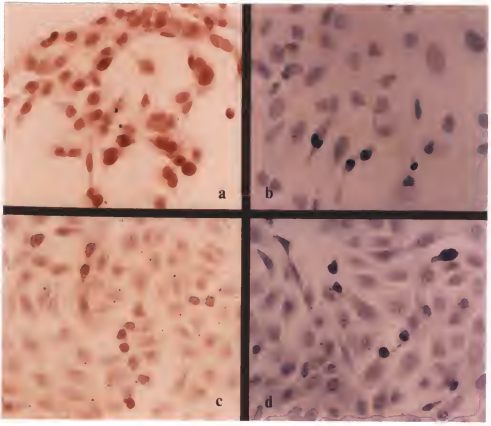

a
b
c
d

Table 4-1. Relative incorporation of [^{3}H]thymidine into DNA of HRE-H9 cells at 33°C and 37°C in the presence of IGF-I, IGF-II, and EGF.

Treatments	[^{3}H]Thymidine incorporation (dpm x 10^{-4})	
	33°C	37°C
PBS	66.7^{a}	15.8^{e}
IGF-I	68.5^{a}	23.5^{c}
IGF-II	68.8^{a}	19.9^{d}
EGF	60.4^{b}	18.0^{de}

Cells received PBS (control) or 100 ng/ml of each growth factor at either 33°C or 37°C and stimulation of [^{3}H]thymidine incorporation was determined (Materials and Methods). Results are means and SE (0.9) of triplicate wells. Means with the same superscript are not significantly different ($\alpha = 0.05$).

effects of temperature ($P < 0.01$) and treatment ($P < 0.01$) and the interaction of temperature x treatment ($P < 0.01$) were detected for DNA synthesis of H9 cells. At 33°C, stimulation of [^{3}H]thymidine incorporation by cells exposed to EGF, IGF-I or IGF-II was not observed. Although EGF inhibited DNA synthesis of cells at 33°C, this was not a consistently observed finding. Since incubations at 37°C and 40°C were equally effective in rendering HRE-H9 cells nontransformed (preliminary observations), experiments were conducted at 37°C at which differential responses to growth factors were demonstrated (Table 4-1).

The dose-dependent mitogenic response of HRE-H9 cells at 37°C to IGF-I is shown in Figure 4-2. IGF-I concentrations up to 10 ng/ml did not stimulate mitogenesis; however, [^{3}H]thymidine incorporation was half-maximal at 34 ng/ml and maximal at 50 ng/ml of IGF-I. Mitogenic responses of cells to increasing doses of

Figure 4-2. Stimulation of DNA synthesis of normal HRE-H9 cells by IGF-I is dose-dependent.

HRE-H9 cells at 37°C were exposed to the indicated amounts of IGF-I in 1 ml of medium and stimulation of incorporation of [^{3}H]thymidine into DNA determined (Materials and Methods). Results are means ± SEM of triplicate wells.

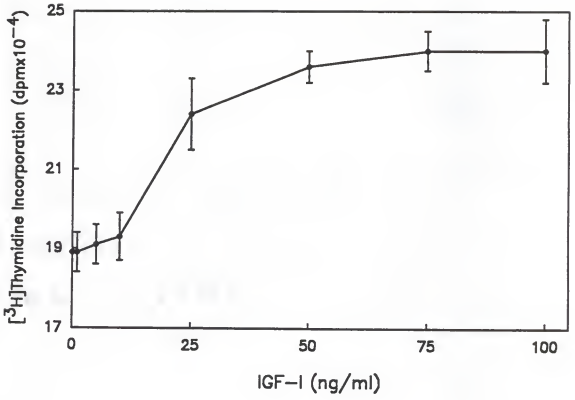
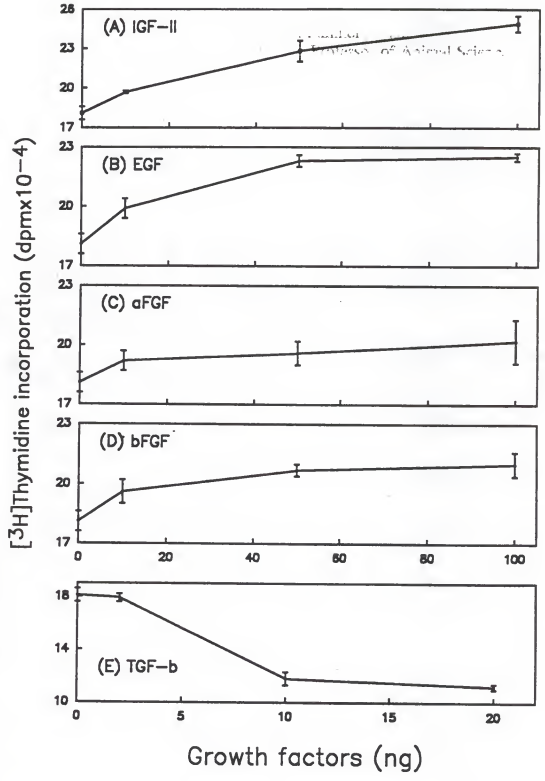

25
23
21
19
17
[3H]Thymidine Incorporation (dpmx10−4)
0
25
50
75
100
IGF−I (ng/ml)

Figure 4-3. Dose-responses of nontransformed HRE-H9 cells to growth factors.

Indicated amounts of IGF-II (A), EGF (B), acidic FGF (C), basic FGF (D), and TGF-β (E) were added to HRE-H9 cells grown at 37°C. After 24 h incubation, mitogen assays were performed to determine growth factor stimulation of [^{3}H]thymidine incorporation into DNA of H9 cells (Materials and Methods). Results are means ± SEM of triplicate wells.


(A) IGF-II
(B) EGF
(C) aFGF
(D) bFGF
(E) TGF-b
[3H]Thymidine incorporation (dpmx10-4)
Growth factors (ng)

other growth factors were also determined. Half-maximal stimulation of DNA synthesis was observed at 42, 29, 32, and 28 ng/ml of IGF-II, EGF, aFGF, and bFGF, respectively, and maximal stimulation at 50-100 ng/ml of growth factors. In contrast, TGF-β1 showed half-maximal inhibition of mitogenesis at 8 ng/ml and maximal at 10 ng/ml (Figure 4-3).

The effects of combinations of growth factors were evaluated (Table 4-2). Acidic and basic FGF had comparable mitogenic activities to EGF, but, when combined with IGF-I, exhibited activity that was lower than for EGF + IGF-I ($P<0.01$). TGF-β1 antagonized the stimulatory effects of growth factors singly or in combinations.

The effects of peptide growth factors on protein synthesis and secretion were also evaluated (Table 4-3). A linear increase in incorporation of [^{3}H]leucine into TCA-precipitable, secreted proteins was observed with increasing incubation time (preliminary observations). Quantitation of [^{3}H]leucine incorporated in CM and CL proteins revealed enhanced cellular protein synthesis in the presence of IGF-I and/or EGF. IGF-I + EGF was most stimulatory in this regard as reflected by the highest levels of [^{3}H]leucine incorporated in CL proteins ($P<0.01$) compared to control. TGF-β1 had no apparent effect on protein synthesis. However, while secretion of de novo synthesized proteins was reduced by IGF-I, EGF, or IGF-I + EGF ($P<0.01$), TGF-β1 increased (by 1.5-fold, $P<0.05$) secretion of these proteins (Table 4-3).

Table 4-2. Effects of growth factors on HRE-H9 DNA synthesis.

Treatments	[^{3}H]Thymidine incorporation (dpm x 10^{-4})
PBS	18.1^{f}
IGF-I	23.2^{d}
IGF-II	21.6^{e}
EGF	21.4^{e}
aFGF	21.6^{e}
bFGF	20.5^{e}
TGF-ß1	12.1^{i}
IGF-I+EGF	31.9^{a}
IGF-I+aFGF	30.3^{b}
IGF-I+bFGF	28.8^{c}
aFGF+bFGF	23.9^{d}
IGF-I+TGF-ß1	12.4^{i}
EGF+TGF-ß1	14.7^{h}
IGF-I+EGF+TGF-ß1	16.9^{g}
IGF-I+aFGF+bFGF	30.6^{b}
aFGF+bFGF+TGF-ß1	12.9^{i}
IGF-I+aFGF+bFGF+EGF	32.5^{a}
IGF-I+aFGF+bFGF+EGF+TGF-ß1	17.0^{fg}

HRE-H9 cells at 37°C were exposed to growth factors (50 ng/ml, except for 10 ng/ml TGF-ß1) in a constant sample volume. Results are means and SE (0.4) of triplicate values. Means with the same superscript are not significantly different ($\alpha = 0.05$).

Table 4-3. Effects of peptide growth factors on incorporation of [^{3}H]leucine into HRE-H9 cell proteins.

Treatments	TCA-precipitable radioactivity (dpm x 10^{-3})	
	CM	CL
PBS	2.7[b]	91[f]
IGF-I	2.0[c]	117[e]
EGF	2.1[c]	120[e]
IGF-I + EGF	2.1[c]	150[d]
TGF-β1	3.3[a]	87[f]

Cells in serum-free, leucine-free medium at 37°C received 10 μCi/ml [^{3}H]leucine and the indicated peptide growth factors (50 ng/ml, except for 10 ng/ml TGF-β1). After incubation for 24 h, conditioned media (CM) and cell lysates (CL) were analyzed for TCA-precipitable radioactivity (20 μl aliquots) (Materials and Methods). Values are means and SE (0.1 for CM and 3.8 for CL) of triplicate wells. Means with the same superscript are not significantly different ($\alpha = 0.05$).

The size and charge distributions of the de novo synthesized and secreted proteins as a function of growth factor treatments were analyzed by 2D SDS-PAGE (Figure 4-4). One to four percent of radiolabeled cellular proteins were secreted during the 24 h incubation period, with a complex profile of radiolabeled proteins in CL (panel B) and CM (panel A). The majority of de novo synthesized and secreted proteins were acidic in nature; however, a number of neutral or weakly basic proteins were also secreted. Proteins exhibiting charge variants were found at approximate M_r of 66,000 in CM and of 40,000, 70,000 and 120,000 in CL, respectively. A CL protein of M_r 45,000 and pI of 5.3 was tentatively identified as actin based on similarity to published 2D-electrophoretic profiles; however, identification of other

radiolabeled proteins was not attempted. Qualitative differences in the 2D-PAGE profiles of [^{3}H]leucine-labeled proteins in CM and CL after addition of IGF-I, EGF, IGF-I + EGF, TGF-ß1, or PBS were not apparent (autoradiograms not shown). Similarly, any quantitative changes in protein profiles by growth factor treatments was not detectable.

The effect of IGF-I on the expression of uteroglobin (UG) mRNA level was examined (Figure 4-5). Using a 430 bp PstI fragment of rabbit UG cDNA, a 600 bp mRNA (Chandra et al., 1981) was detected by a Northern blot analysis, but expression of its message was not affected by IGF-I treatment.

To identify the receptor(s) on HRE-H9 cells responsible for the mitogenic actions of the IGFs, affinity cross-linking of iodinated IGFs to cell monolayers grown at 33°C and 37°C was performed (Figure 4-6). A labeled diffuse band of M_r 130,000, representing IGF-I cross-linked to the α-subunit of the Type I receptor, was detected after SDS-PAGE and autoradiography of solubilized cells previously grown at either temperature (panels A: 37°C and B: 33°C). Higher M_r cross-linked complexes were also apparent at 37°C. Incubation with unlabelled IGF-I or IGF-II (50 ng/ml), but not insulin (50 ng/ml), completely inhibited the formation of the cross-linked complexes. Interestingly, cross-linked complexes representing [^{125}I]IGF-II bound to receptors were not apparent for these cells at either temperature (panel C at 37°C and D at 33°C), although IGF-II was nearly equipotent to IGF-I in mitogenic activity.

Figure 4-4. Electrophoresis of conditioned medium and cell lysate from IGF-I-treated HRE-H9 cells.

Cells were cultured in serum-free, leucine-free MEM at 37°C for 24 h in the presence of IGF-I (50 ng/ml) and 50 μCi/ml [^{3}H]leucine, and CM and CL were prepared for 2D-SDS-PAGE (Materials and Methods). After dialysis, 100,000 cpm of CM (A) and 150,000 cpm of CL (B) were subjected to IEF (pH 3.5-10) followed by 10% SDS-PAGE under reducing conditions. Gels were treated with sodium salicylate before drying and fluorography (2 day exposure for CL and 7 day exposure for CM).

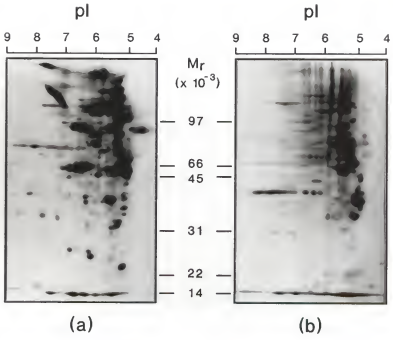

pI
9 8 7 6 5 4
pI
9 8 7 6 5 4
Mr
(x 10^-3)
97
66
45
31
22
14
(a)
(b)

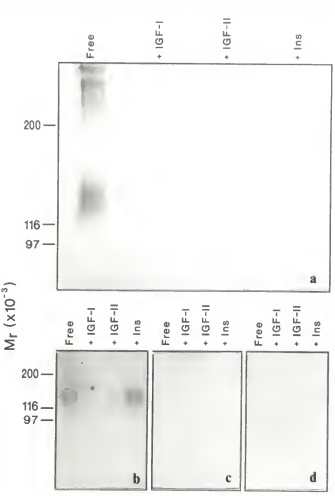
Figure 4-5. Northern blot analysis of the expression of uteroglobin (UG) mRNA in normal HRE-H9 cells.

Nontransformed H9 cells were grown to confluency at 37°C, changed to serum-free MEM, and incubated for 24 h in the presence or absence of IGF-I (25 ng/ml). Forty microgram of total cellular RNA was isolated and hybridized with a 430 bp PstI fragment of rabbit UG cDNA (Materials and Methods). A 600 bp UG mRNA was detected, but its expression was not affected by IGF-I.


IGF-I
(−)(+)
◄UG (600 bp)

Figure 4-6. Affinity cross-linking of [^{125}I]IGF-I and [^{125}I]IGF-II to HRE-H9 monolayers.

Confluent HRE-H9 cells at 37°C (A, C) and 33°C (B, D) were incubated with 500,000 cpm (0.2 nM) [^{125}I]IGF-I (A, B) or [^{125}I]IGF-II (C, D) in the absence (shown as free) and presence of unlabeled competitor peptides (50 ng/ml) as indicated. After incubation for 16 h at 4°C, cell monolayers were treated with DSS. Cell lysates were analyzed by SDS-PAGE and autoradiography.


Free
+ IGF-I
+ IGF-II
+ Ins
200
116
97
a
M_r ($x10^{-3}$)
Free
+ IGF-I
+ IGF-II
+ Ins
200
116
97
b
c
d

The presence of IGF binding proteins (IGFBPs) in CM and CL from HRE-H9 cells cultured at 33°C and 37°C with and without phenol red (weak estrogen) in culture media was examined by ligand blot analysis (Figure 4-7). Phenol red in culture medium had no discernible effect on IGFBP levels. Increased levels of IGFBPs were observed when cells were cultured at 37°C than at 33°C. A predominant band of M_r 31,000 was detected in CM at 37°C and in all CLs evaluated. An IGFBP of M_r 35,000 was detected only at 33°C; however, an IGFBP of M_r 27,000 was present in CLs at 33°C and 37°C.

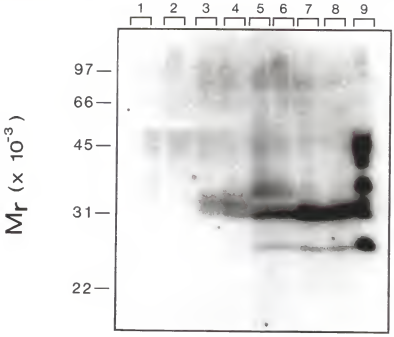
Discussion

This study examined the effects of several peptide growth factors on the proliferation and protein synthesis and secretion of the rabbit endometrial epithelial cell line, HRE-H9, during normal and transformed states. This continuous cell line retains characteristics of the tissue of origin which include secretion of β-endorphin (Li et al., 1989) and maintenance of transcriptional activity for uterine-specific genes (Fliss et al., 1991b).

Nontransformed HRE-H9 cells at 37°C responded to growth factors in a dose-dependent manner, whereas transformed HRE-H9 cells (33°C) were refractory to these proteins. The absence of in vitro reponses to IGFs by transformed HRE-H9 cells contrasted with their expression of Type I IGF receptors and low but detectable synthesis of IGFBPs. Since an autocrine mode of growth factor action on

Figure 4-7. IGFBPs in conditioned media and cell lysates.

Cells were incubated in serum-free MEM for 24 h, and CM and CL were subjected to ligand blot analysis using [^{125}]IGF-II (Materials and Methods). Odd numbers represent CM and CL obtained from cells in phenol red-free MEM and even numbers from cells in MEM with phenol red, respectively. CM (lanes 1 and 2) of cells grown at 33°C, and CM (lanes 3 and 4) of cells at 37°C, CL (lanes 5 and 6) of cells at 33°C, and CL (lanes 7 and 8) of cells at 37°C were compared with M_r standards and bovine neonatal serum (lane 9), a positive control for IGFBPs.


1 2 3 4 5 6 7 8 9
97
66
45
31
22
Mr (x 10-3)

transformed cells is well established (Sporn and Roberts, 1985, Simmen and Simmen, 1991), a similar mechanism may account for the lack of response of HRE-H9 cells to exogenous growth factors. This possibility is consistent with their reduced requirement for serum (Sporn and Roberts, 1985) and with our studies showing that growth rates of transformed HRE-H9 cells in 1% or 5% serum-containing media are comparable. The response of nontransformed HRE-H9 cells to added IGFs agrees with previous studies documenting mitogenic effects of IGFs on various cell types including epithelia. However, while both IGF-I and IGF-II were mitogenic for HRE-H9 cells, epithelial cell lines derived from other tissues were only responsive to IGF-I (Falco et al., 1988; Mouzon and Kahn, 1991). In contrast to the identification of Type I IGF receptors using [^{125}I]IGF-I, Type I or II IGF receptors were not detected using [^{125}I]IGF-II as ligand. The relative absence of Type II receptors on these cells and/or the 3-5-fold lower affinity for binding of IGF-II than IGF-I to Type I IGF receptors (Clemmons, 1989; Humbel, 1990) may account for this result. That the IGF-II tracer used was biologically active is suggested by its ability to bind to IGFBPs. The intensities of all bands observed after cross-linking with [^{125}I]IGF-I were partially diminished by excess unlabeled insulin which also supports their correspondence to the Type I receptor. These results, therefore, suggest that the mitogenic actions of both IGF-I and IGF-II in HRE-H9 cells are mediated via the Type I receptor tyrosine kinase. This is similar to the IGF ligand-receptor system operative in certain mammary epithelial cell lines (Osborne et al., 1989).

In vivo, IGF-I is synthesized by uterine endometrium and myometrium, tissues which also co-express IGF receptors (Hofig et al., 1991a). IGF-I was shown to stimulate proliferation of uterine endometrial stromal cells in culture (Chapter 2; Ghahary et al., 1990). In the present study of uterine epithelial cells, IGF-I had the highest activity among the growth factors evaluated and, in addition, potentiated the effects of other stimulatory growth factors. Thus, uterine epithelium and stroma are likely in vivo target tissues for IGF-I, potentially of myometrial, endometrial and blood origins.

The interactions of EGF with IGF-I and IGF-II, as observed in the present study, are likely to occur in vivo. EGF is a mitogen for uterine cells of several species (Gerschenson et al., 1979; Tomooka et al., 1986; Huet-Hudson et al., 1990; Irwin et al., 1991). Localization of EGF receptors on mouse and pig embryos (Paria and Dey, 1990; Corps et al., 1990) and human uterine epithelium (Smith et al., 1991) suggests roles for EGF or EGF-related peptides in embryonic and uterine growth during early pregnancy. The present results demonstrating mitogenic activities of a/bFGFs contrasts with the reported absence of an FGF mitogenic effect on immature mouse uterine epithelial cells in vitro (Tomooka et al., 1986). Moreover, human endometrial stromal cells in the presence of progesterone were growth-stimulated by bFGF, with an activity comparable to that of EGF (Irwin et al., 1991). Since FGFs are apparently synthesized in the uterus (Brigstock et al., 1990), these factors are likely to interact with IGF-I, EGF and other growth factors to mediate uterine growth and/or differentiation.

Mitogenesis of HRE-H9 cells was inhibited by TGF-ß1, a known inhibitor of epithelial cell proliferation (Edwards and Heath, 1991). The signal transduction mechanism for the anti-proliferative actions of TGF-β has not been completely elucidated. However, since TGF-ß is a regulator of expression of protooncogenes at the transcriptional and/or post-transcriptional levels, TGF-ß-induced growth inhibition may be due in part to suppressed expression of protooncogenes (Edwards and Heath, 1991). TGF-ß1 has been shown to maintain the product of the retinoblastoma tumor suppressor gene ($p105^{Rb}$) (Laiho et al., 1990) in an active form which can act as a negative regulator of c-fos (Robbins et al., 1990) and c-myc (Pietenpol et al., 1990) gene transcription. The induction by IGF-I of expression of protooncogenes c-fos and c-myc (Mouzon and Kahn, 1991) and the observation that cells transfected with c-myc and c-ras become resistant to growth inhibitors (Strom et al., 1991) also support the involvement of protooncogenes in growth inhibition. Alternatively, the ability of TGF-ß to down-regulate receptors for EGF in rat fibroblasts, but not in mink lung epithelial cells (Like and Massague, 1986) suggests an apparent cell type-specific transmodulation by TGF-ß of receptors for other ligands. It is possible that the binding of TGF-ß1 to its cell surface receptors decreased the binding affinities of other receptors for their respective ligands. Similarly, transmodulation has been reported for PDGF and EGF receptors (Bowen-Pope et al., 1983) and for a/bFGF and EGF receptors (Hicks et al., 1989).

Although IGF-I and EGF stimulated protein synthesis similar to their effects on DNA synthesis, they did not stimulate secretion of de novo synthesized proteins by

H9 cells. IGF-I stimulated protein synthesis and inhibited protein degradation of muscle cells in culture, suggesting multifunctional roles for growth factors in protein turnover and secretion (Vandenburgh et al., 1991). Although TGF-β1 inhibited growth of HRE-H9 cells, its stimulatory effect on secretion of de novo synthesized proteins may be related to its role as a mediator of cell differentiation (Donjacour and Cunha, 1991). TGF-ß has been shown to affect the extracellular environment of cells by regulating the synthesis, deposition and turnover of extracellular matrix proteins and cell adhesion molecules (Edwards and Heath, 1991). In the present study, effects of this growth factor or all growth factors on relative labeling of individual secreted proteins were not apparent. Mulholland et al. (1988) reported that the overall profile of proteins synthesized by rabbit endometrial epithelial cells was unchanged in primary culture regardless of hormonal (progesterone, estradiol, prolactin, or combination) treatments. Profiles of endometrial proteins produced in vitro have been published (Mulholland et al., 1988; Takeda et al., 1988; Santoro et al., 1989); however, it is difficult to compare these with the 2D-PAGE profile for HRE-H9 cells, since uterine cell types of different species produce distinct classes of proteins (this study, Mulholland et al., 1988; Takeda et al., 1988; Santoro et al., 1989).

Among the uterine-expressed IGFBPs, IGFBP-1, which is identical to placental protein 12 (Rutanen et al., 1986) and pregnancy-associated endometrial secretory α_1-globulin (α_1-PEG) (Waites et al., 1988), with an estimated M_r of 29,000-32,000 is the best characterized. The presence of a putative IGFBP-1 (M_r of 31,000) in lysates and

culture medium of tranformed and non-transformed HRE-H9 cells is consistent with endometrial expression of this protein. IGFBP-1 mRNA is synthesized by uterine epithelial and stromal cells (Rutanen et al., 1986; Croze et al., 1990; Julkunen et al., 1990). In addition, use of a monoclonal antibody to α_1-PEG detected this protein, specifically in the luminal epithelium of pregnant sheep uterus (Waites et al., 1990).

The physiological significance of the suppression of HRE-H9 IGFBP production and secretion during transformation is yet to be determined. The overall higher levels of IGFBPs at 37°C than at 33°C could account for the lower basal mitotic activity of HRE-H9 cells at 37°C than at 33°C. This is consistent with an inhibitory role of IGFBP-1 on IGF-mediated events (Ritvos et al., 1988a). In fact, when radioimmunoassay was used to quantitate IGF-I levels in media conditioned by cells cultured in the presence and absence of phenol red at 33°C and 37°C, the net production of IGF-I per cellular DNA contents was higher in CM by cells cultured at 33°C compared to 37°C (9.02 ± 0.22 vs. 4.88 ± 0.54 pg/μg DNA). However, the effects of phenol red on IGF-I secretion by HRE-H9 cells were not detected. Alternatively, a possible autocrine mode of IGF action on transformed HRE-H9 cells may lead to inhibition of binding protein expression and a higher basal mitotic rate of cells. The observation that insulin inhibits the expression of IGFBP-1 in human (Conover and Lee, 1990) and rat (Orlowski et al., 1991) hepatoma cells is consistent with this possibility. It is of interest to examine other transformed cell lines (Chou, 1989) for altered production of IGFBPs, in particular to determine if this is a general aspect of the SV40-mediated transformation process. Reductions in expression levels

of inhibitory IGFBPs and/or increases in production of stimulatory IGFBPs may generally contribute to the transformed phenotype. The secretion of IGFBP-1 as well as other IGFBPs by the HRE-H9 cell line suggests its potential use in future studies to elucidate the steroidal and endometrial epithelial-specific regulation of IGFBP production.

In summary, the mitogenic effects of IGFs -I and -II on HRE-H9 cells are likely to be mediated primarily by Type I IGF receptors. EGF is also mitogenic for these uterine epithelial cells, alone and in combination with the IGFs. TGF-β1, in contrast, is an inhibitor of HRE-H9 cell growth and antagonizes the stimulatory effects of the other growth factors. IGFBPs are synthesized, in general, at higher levels in nontransformed than in transformed HRE-H9 cells and normally may serve to mediate the autocrine and paracrine actions of IGFs on uterine epithelium. Growth factors regulated protein synthesis and secretion, although qualitative differences in the types of proteins synthesized by this differentiated cell type were not apparent. These results point to the complex interactions that are likely to occur in vivo among stimulatory and inhibitory growth factors to modulate endometrial epithelial mitogenesis and protein synthesis and secretion during the estrous cycle, implantation and fetal development.

CHAPTER 5
SUMMARY AND CONCLUSIONS

Summary of Results

A review of the current understanding and state of research regarding prenatal mortality in pigs, maternal recognition of pregnancy, uterine secretory proteins, and uterine-derived growth factors was presented in Chapter 1. The synchronous development of uterus and conceptuses during the peri-implantation period is considered to be critical for successful pregnancy. This coordinate tissue development is accomplished through maternal-fetal communication via the involvement of protein mediators, such as peptide growth factors, as hypothesized for the experimental design of the present studies.

Recent interest in peptide growth factors and the role of uterine secretions in conceptus development was responsible for the search for and characterization of growth promoting factors in porcine uterine luminal fluids. The partial purification and initial characterization of a growth factor in porcine uterine luminal fluids, termed uterine luminal fluid mitogen (ULFM), was described in Chapter 2. Results obtained from this study are summarized as follows: (1) ULFM is a small M_r polypeptide with a M_r of 4,800 and a pI of about 6.4 that is inactivated by trypsin, proteinase K and ß-mercaptoethanol, but heat-stable; (2) ULFM is not a pregnancy-

specific protein since a similar mitogenic factor was present in ULFs from cyclic gilts; (3) ULFM is apparently distinct from other known peptide growth factors, such as IGF-I, IGF-II, EGF, TGFs, FGFs, PDGF, and CSF-1, which suggests that ULFM is a novel mitogen; and (4) partially purified ULFM is a potent mitogen for both fibroblastic and epithelial cells with activities comparable to those for EGF and IGF-I in proliferation and mitogen assays utilizing both cell types.

The identification of a mitogen distinct from known growth factors in porcine ULF led to the studies of sheep ULF growth factors and comparisons with porcine ULF, since both sheep and pig conceptuses are characterized by rapid morphological changes during the peri-implantation period of development and both have noninvasive placentation. The results of this study (Chapter 3) are summarized as follows: (1) ULFs from cyclic and pregnant ewes contain both IGF-I and IGF-II. ULF IGF-I levels for cyclic ewes were higher than those for pregnant ewes and changes were affected by day in cyclic and pregnant ewes. ULF IGF-II content was not different between cyclic and pregnant ewes, but IGF-II levels varied depending on the day of the estrous cycle or pregnancy. IGF-II levels were generally higher than IGF-I levels in the ovine ULFs examined; (2) Ovine ULFs contain mitogenic factor(s) distinct from the IGFs. A gradual increase in mitogen activity from Day 10 to 14 and a decrease on Day 16 were observed for ULFs from both cyclic and pregnant ewes. The mitogenic activity in the ULFs from cyclic ewes was heat-sensitive, but the mitogenic activity in ULFs from pregnant ewes at each day of pregnancy was differentially affected by heat-treatment, showing a greater percentage

reduction for those ULFs with higher mitogenic activity. Overall, the temporal variation in AKR-2B mitogenic activity in ULFs mimicked the developmental profile for ULF IGF-II in cyclic and pregnant ewes and for ULF IGF-I in pregnant ewes; (3) Ovine ULFs do not contain PDGF or porcine ULFM-like activity. The differences in M_r and in degree of inactivation by heat-treatment of the ovine and porcine mitogenic factor(s) suggested the absence of ULFM in ovine ULFs; (4) The secretion of ovine trophoblast protein-1 (oTP-1) was stimulated or at least maintained by the combination of IGF-I and IGF-II, but not by IGF-I or IGF-II alone. The more advanced conceptuses, as judged by their size and morphology, secreted more oTP-1; and (5) ULFs from cyclic and pregnant ewes contained at least four different mitogenic factors, namely, IGF-I, IGF-II and two additional, as yet unidentified, mitogenic factors. Results of this study also indicated a possible regulatory role for IGFs in synthesis and/or secretion of oTP-1 during the period of maternal recognition of pregnancy in sheep.

The results presented in Chapters 2 and 3 indicate the presence of several growth factors in uterine secretions of domestic species. Since peptide growth factors are multifunctional, subsequent interest was focused on their actions and interactions during uterine cell growth and differentiation. The studies described in Chapter 4 were initiated to examine the in vitro effects of peptide growth factors identified in the uterus focusing in particular on DNA and protein synthesis. Rabbit endometrial epithelial cells (HRE-H9) transformed with an origin-defective, temperature-sensitive SV40 mutant at 33°C were used since this cell line retains characteristics of the

tissue of origin, but with an unlimited life span. At present, there are no established endometrial epithelial cell lines from any domestic species. Results of this study are summarized as follows: (1) Peptide growth factors, such as IGF-I, IGF-II, EGF, and FGFs, stimulated DNA synthesis of non-transformed cells in a dose-dependent manner, but not that of transformed cells. This result supports the autocrine mode of growth factor action to render cells transformed and possible in vivo regulation by these growth factors of normal uterine endometrial epithelial cell proliferation; (2) TGF-β1 inhibited uterine epithelial cell growth, a result that indirectly confirmed the epithelial nature of the HRE-H9 cell line; (3) TGF-β1 stimulated protein secretion, probably due to its stimulatory effects on synthesis, deposition, and turnover of extracellular matrix proteins and cell adhesion molecules; (4) The synergistic and antagonistic interactions of growth factors during uterine epithelial cell growth were also demonstrated. The most stimulatory effects on DNA and protein synthesis were the combination of IGF-I and EGF, effects abrogated by TGF-β1; (5) The mitogenic actions of IGF-I and IGF-II on HRE-H9 cells were shown to be mediated by Type I IGF receptors because of the relative absence of Type II IGF receptors on these cells; and (6) The presence of IGFBPs in cell lysates and culture medium of HRE-H9 cells was confirmed. A putative IGFBP-1 with a M_r of 31,000 was secreted. The higher levels of IGFBPs synthesized and secreted at 37°C than at 33°C may account for the lower basal mitotic activity of HRE-H9 cells at 37°C than at 33°C, as IGFBP-1 is generally inhibitory in IGF-mediated physiological processes. Overall, results of this study demonstrated the complex

interactions that are likely to occur in vivo among stimulatory and inhibitory growth factors to modulate the growth and differentiation of endometrial epithelial cells during the estrous cycle and pregnancy.

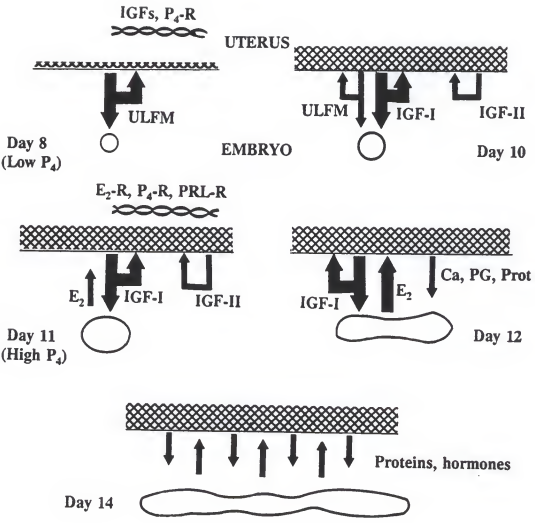
Conclusions

Embryonic development is regulated by endocrine, genetic, and biochemical factors. The synchronous development between the dam and the early embryos appears to be a major determining factor for the developmental fate of embryos. The importance of peptide growth factors in uterine secretions for the regulation of uterine and conceptus development has been previously documented. Further, it is important to understand the developmental biology of the conceptus and uterus in order to define the molecular basis for embryonic losses. Based on the information available and the results of the present studies, a regulatory pathway for coordinate conceptus and uterine development involving peptide growth factors during early pregnancy in pigs is proposed (Figure 5-1).

Around Day 8 of pregnancy when serum progesterone levels are low, ULFM is actively secreted from uterine endometrium into uterine lumen, where it acts on uterus and conceptuses via autocrine and/or paracrine routes for their proliferation and differentiation. Further, ULFM may upregulate transcriptional activity of other growth factor genes, such as IGF-I. As serum progesterone levels rise around Day 10, ULFM activity decreases and the progesterone-dependent endometrial IGF-I

Figure 5-1. A model for a possible mechanism of peptide growth factors in the regulation of conceptus and uterine development during early pregnancy in the pig.

At developmental stage, thick lines represent the major regulatory factors and arrows indicate the direction of secretion and action of those factors


IGFs, P_4-R
UTERUS
ULFM
Day 8
(Low P_4)
EMBRYO
ULFM
IGF-I
IGF-II
Day 10
E_2-R, P_4-R, PRL-R
E_2
IGF-I
IGF-II
Day 11
(High P_4)
IGF-I
E_2
Ca, PG, Prot
Day 12
Proteins, hormones
Day 14

becomes a major secretory molecule which stimulates coordinate development of conceptus and uterus by binding to cell surface receptors. IGF-I may stimulate synthesis of endometrial receptors for other factors, such as progesterone, in preparation for enhanced uterine secretory activity. Additionally, IGF-I in combination with IGF-II may modulate the metabolic activity of conceptuses, such as aromatase activity, which results in the stimulation of estrogen synthesis and secretion by Day 11-12 conceptuses. The secretion of estrogens causes release of several endometrial regulatory molecules into uterine lumen, resulting in maternal recognition of pregnancy in the pig. These secreted molecules are also involved in the regulation of other reproductive functions, such as uterine development, implantation, and possibly embryonic mortality. As serum progesterone levels begin to decrease around Day 14 of pregnancy, the expression of endometrial IGF-I mRNA and proteins decreases accordingly.

In order to verify this model, the following evidences and discussions are presented. Uterine functions are under the control of steroids estrogens and progesterone (Chapter 1). During the estrous cycle in the pig, circulating progesterone levels are low at estrus, begin to increase rapidly after Day 2, reach peak values around Days 10-12, and then decline rapidly thereafter until next ovulation. In contrast, systemic estrogen concentrations begin to increase coincidently when progesterone levels start to decline (Anderson, 1987). The observation in Chapter 2 that ULFM is not a pregnancy-specific, uterine-derived mitogen suggests the regulation of ULFM activity by both steroids. ULFM activity

is the highest on Day 8 of pregnancy and there is a decrease in ULFM activity between Day 8 and 14 of the estrous cycle in the pig (Simmen et al., 1989a), which is coincident with the increase of progesterone levels. Since estrogen levels are constantly low during this period, the synthesis and secretion of ULFM may be negatively regulated by progesterone. The similar inhibition by progesterone of ULFM activity is also postulated for pregnant gilts (Simmen et al., 1989a).

ULFM stimulated DNA synthesis of both epithelial and embryo-derived fibroblastic cells (Chapter 2), indicating the possible regulatory roles for ULFM in embryonic cell proliferation. In this regard, the binding of ULFM to embryonic cells may directly stimulate embryonic growth. Indirectly, ULFM may induce the secretion of other growth factors and/or sensitize the cells for binding of other growth factors, all of which would result in augmented embryo growth. ULFM was also found to stimulate DNA synthesis of uterine stromal fibroblastic cells (Chapter 2). Thus, ULFM may directly stimulate uterine growth via autocrine/paracrine routes. Further, ULFM action on stromal cells may regulate transcription of genes encoding other growth factors, such as IGF-I, for synergistic effects on growth. ULFM may also induce progesterone receptors in uterine endometrium in lieu of rising concentrations of circulating progesterone. All of these postulated events of ULFM would result in augmented uterine and embryo growth.

High concentrations of progesterone at mid-cycle result in increasing endometrial secretory activity (Roberts and Bazer, 1988). One identified uterine secretory product around this period is IGF-I whose maximal endometrial synthesis

immediately follows the decline in ULFM in ULFs (Simmen and Simmen. 1990). IGF-I is a mitogen and differentiation factor which binds to cell surface receptors on various cell types and functions via autocrine and/or paracrine routes (Zapf and Froesch, 1986). Contrary to ULFM, the endometrial expression of IGF-I mRNAs is positively related to systemic progesterone levels (Simmen and Simmen, 1990). Cyclic pigs exhibited peak levels of IGF-I in the lumen on Days 10-12 of the estrous cycle (Simmen et al., 1989a). In pregnant gilts, the highest levels of IGF-I mRNA in endometrium and of IGF-I in conceptus and the uterine lumen were observed on Day 12 of gestation (Letcher et al., 1989a; Simmen et al., 1990). Binding sites for IGF-I are expressed on the pig blastocyst and in uterine endometrium and myometrium (Corps et al., 1990; Hofig et al., 1991a).

The binding of IGF-I to its receptors on pig conceptuses results in significant changes in the uterine environment. IGF-I was demonstrated to stimulate protein synthesis and secretion by pig embryonic discs in vitro (Estrada et al., 1991) and aromatase P450 activity of porcine conceptuses (Hofig et al., 1991b). Therefore, it is likely that IGF-I in the lumen is bound by conceptuses, probably via IGF receptors, and stimulates conceptus growth and differentiation. Enhanced aromatase activity may result in synthesis and secretion of estrogens into the uterine lumen. The conceptus-derived estrogens are known to stimulate the release of calcium, $PGF_2\alpha$, and other secretory proteins by the uterus into the uterine lumen (Zavy et al., 1980; Geisert et al., 1982b; Godkin et al., 1982a; Fazleabas et al., 1983; Simmen and Simmen 1990). Estrogens are also the signal for the maternal recognition of

pregnancy in pigs (Bazer and Thatcher, 1977). These sequential events, triggered by IGF-I, leading to estrogen secretion by pig conceptuses indicate a possible regulatory role for IGF-I in maternal recognition of pregnancy in pigs. However, IGF-II is thought to be a mediator of this process, since the possible synergism and/or antagonism of IGF-I with IGF-II may be critical for conceptus secretion of estrogens. Greater levels of endometrial IGF-II mRNAs were observed for the less prolific European Large White (LW) than the prolific Chinese Meishan (MS) gilts at Day 12 of pregnancy, whereas ULF IGF-I levels between the two breeds at the same day of pregnancy were the opposite (Simmen et al., 1992). Additionally, greater amount of estrogens were observed in ULFs from MS than from LW gilts (Bazer et al., 1991b) and IGF-II was shown to inhibit human placental P450 aromatase activity (Nestler, 1990). The ratio of IGF-I to IGF-II in the ULFs may therefore determine, in part, the conceptus secretory activity during maternal recognition of pregnancy (Simmen et al., 1992). This temporal correlation between IGF-I and IGF-II and conceptus secretory activity is also likely to be important in other domestic animal species. The combination of IGF-I and IGF-II stimulated or at least maintained the secretion of ovine trophoblast protein-1, the signal for maternal recognition of pregnancy in sheep (Chapter 3). But, greater levels of ULF IGF-II than IGF-I were observed during this period in sheep (Chapter 3), supporting an inhibitory role for IGF-II in conceptus estrogen production in ruminants. Similarly, the secretion of bovine trophoblast protein-1 during the period of maternal recognition of pregnancy in cows is coincident with excess IGF-II over IGF-I in ULFs (Geisert et al., 1991).

The synergistic interactions of growth factors may be necessary to stimulate uterine growth, since sufficient intrauterine space is required to accommodate rapidly elongating conceptuses. In this regard, IGF-II was shown to stimulate DNA synthesis of endometrial epithelial cells and IGF-I potentiated these IGF-II actions (Chapter 4). Additionally, IGF-I was demonstrated to stimulate DNA synthesis of uterine endometrial stromal cells (Chapter 2). Thus, IGF-I may, via autocrine and paracrine routes, stimulate uterine growth and differentiation correspondent with conceptus growth and secretion of maternal recognition of pregnancy molecules. Peptide growth factors may also stimulate synthesis of receptors for estrogen, prolactin, and progesterone (Sumida et al., 1988; Aronica and Katzenellenbogen, 1991) in endometrial cells to enhance uterine secretory activity responses to these circulating hormones.

The circulating progesterone levels begin to decline around Day 14 of pregnancy. Thus, progesterone-dependent expression of IGF-I results in decreased IGF-I mRNA levels in endometrium (Letcher et al., 1989a). Accordingly, IGF-I content in the uterine lumen also decreases (Simmen and Simmen, 1990).

This proposed model does not rule out the possible involvement of other endocrine, genetic, and nutritional factors in coordinate uterine and conceptus development. Although several aspects of the proposed pathway remain to be more precisely elucidated, an attempt was made to identify roles for ULFM and IGFs in the regulation of conceptus and uterine development during early pregnancy in pigs.

In summary, the studies in Chapters 2-4 have identified peptide growth factors in the uterine secretions of domestic species and have demonstrated their effects on conceptus and endometrial functions, and their synergistic and/or antagonistic interactions with regard to endometrial epithelial cell growth. Based on this information, a model was proposed which related progesterone, ULFM, and IGFs as mediators of coordinated conceptus and uterine development during early pregnancy. Future studies regarding the structure and functions of ULFM, the possible interactions of ULFM and the IGFs with estrogens and/or progesterone, and the in vivo effects of growth factors on conceptus and endometrial activities are required to critically examine the validity of the proposed model. This in turn may provide clues as to the basis for prenatal mortality during early pregnancy in the domestic animal species and lead to development of rational, biotechnology-based schemes for alleviating this significant reproductive problem.

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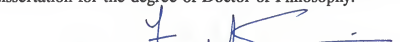
BIOGRAPHICAL SKETCH

Yong Ko was born in Kwang-Ju, Korea, on May 7, 1957. He attended Korea University in Seoul and earned a Bachelor of Science degree in animal science in 1980. After completing service in the Army in 1983, he entered the graduate program in the Animal Science Department at The Ohio State University. He worked under the guidance of Dr. Walter R. Threlfall and earned a Master of Science degree in the area of reproductive physiology in March 1986. Then, he continued to pursue his doctoral program with Dr. Frank A. Simmen at the same department. In May 1989, along with Dr. Frank A. Simmen, he joined the Dairy Science Department at the University of Florida and completed the requirements for the degree of Doctor of Philosophy in May 1992. He is going to work for Dr. Frank A. Simmen as a postdoctoral fellow in the Dairy Science Department at the University of Florida in Gainesville, Florida.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



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